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Sesamin suppresses aging phenotypes in adult muscular and nervous systems and intestines in a *Drosophila* senescence-accelerated model

T.D. LE[1], Y. NAKAHARA[1], M. UEDA[1], K. OKUMURA[1], J. HIRAI[1], Y. SATO[2], D. TAKEMOTO[2], N. TOMIMORI[2], Y. ONO[2], M. NAKAI[2], H. SHIBATA[2], Y.H. INOUE[1]

[1]Insect Biomedical Research Centre, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto, Japan
[2]Institute for Health Care Science, Suntory Wellness Ltd., Seika-cho, Soraku-gun, Kyoto, Japan

Tuan Dat Le and Yasuyuki Nakahara contributed equally to this work

Abstract. – OBJECTIVE: Sesamin is a major lignan constituent of sesame and possesses various health-promoting effects. Previous studies have demonstrated that sesamin extends the lifespan of *Drosophila* and *Caenorhabditis elegans* and corrects oxidative damage-related tissue dysfunction in mammals. To understand its anti-aging effects, we aimed to determine whether sesamin restores tissue function hampered by oxidative damage and suppresses several aging-related phenotypes using *Drosophila* senescence-accelerated models.

MATERIALS AND METHODS: We elucidated the anti-aging effects of sesamin on several aging-related phenotypes in the muscle, brain and midgut using the senescence-accelerated models (*Sod1*^{n1} mutant and *Sod1*-depleted flies) by immunostaining experiments. We determined the expression levels of several anti-oxidative and DNA repair genes using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). We also identified the metabolite of sesamin in *Drosophila* by LC-MS/MS.

RESULTS: We confirmed that sesamin (0.35 and 2 mg/ml) extended the lifespan of the fly models. As observed in mammals, it can be absorbed and metabolized by *Drosophila* adults. The sesamin feeding suppressed the age-dependent impairment of locomotor activity and inhibited the accumulation of reactive oxygen species (ROS) in their bodies. Sesamin delayed the age-dependent accumulation of damaged proteins in the muscle, partially suppressed the loss of dopaminergic neurons in adult brains displaying ROS accumulation, and suppressed the accumulation of DNA damage and hyperproliferation of intestinal stem cells. Four antioxidative genes and two DNA repair genes were simultaneously upregulated in sesamin-fed adults.

CONCLUSIONS: These observations represent the first direct evidence of the anti-aging effects of sesamin at the individual level. We propose that sesamin exerts anti-aging effects in the muscles, brain and midgut by inducing antioxidative and DNA repair genes, resulting in extended lifespan in flies.

Key Words:
Sesamin, Anti-aging, *Drosophila*, Muscle, Intestine.

Introduction

Sesame seeds contain certain types of lignans that play important roles in health promotion[1]. Sesamin is a major lignan in sesame seeds. Many *in vitro* and *in vivo* studies have demonstrated its biological effects, including antioxidant[2-6], anti-carcinogenic[7,8] and anti-hypertensive effects[9-11]. Thus, sesame seeds have been used as a traditional health food as well as a medicinal plant, consumed as a supplement or remedy. After intake, sesamin is metabolized to SC1, (1R,2S,5R,6S)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0] octane, by cytochrome P450 in mammals[3,12]. Because SC1 displays potent antioxidant activity, it may be responsible, in part, for some of the physiological activities of sesamin[3,13,14]. A previous study[10,15] using experimental animals has demonstrated the anti-hypertensive effects of sesamin and has suggested that sesamin feeding prevents hypertension by inhibiting NADPH oxidase activity and the expression of its subunits. Another study[3] has reported that the catechol moiety of the sesamin molecule is responsible for the protective effects of sesamin against oxidative damage in the rat liver. It has recently been reported

Corresponding Author: Yoshihiro H. Inoue, MD; e-mail: yhinoue@kit.ac.jp

that sesamin extends the mean lifespan and alleviates paraquat-induced decline in the survival rate of *Drosophila* by interacting with antioxidant enzymes[16]. The compound also enhances host defense and increases the average lifespan of *Caenorhabditis elegans*[17]. Although there is much biological and biochemical evidence demonstrating the anti-aging effects of sesamin in animals, a few studies have aimed to verify its role in hindering the aging process. Apart from simply measuring the lifespan of organisms, it is not easy to monitor the progression of aging and evaluate the anti-aging effects simultaneously. The fruit fly *Drosophila melanogaster* serves as a useful genetic model due to its high fecundity and short life cycle[18,19]. Furthermore, advances in genetic techniques have facilitated the experimental investigation. *Drosophila* has numerous advantages in drug discovery[18,20], particularly as an experimental model for studies on aging. We previously established an experimental system that allowed the downregulation of the *Sod1* gene encoding the Cu/Zn superoxide dismutase that eliminates superoxide radicals among reactive oxygen species (ROS)[21,22]. As normal *Drosophila* adults age, they display impaired locomotor activity, accumulation of abnormal protein aggregates containing polyubiquitinated proteins in the muscle and loss of dopaminergic (DA) neurons in the brain[22-25]. Moreover, as the flies' ages increase, intestinal stem cells increase in number in intestinal epithelial cells[26-28]. We showed that adults with depleted *Sod1* and its hypomorphic mutant, $Sod1^{n1}$, exhibit not only a shortened lifespan but also accelerated age-dependent phenotypes from an earlier stage. Therefore, compared with mouse and rat models, these flies represent a good senescence-accelerated model for the rapid evaluation of anti-aging effects[22]. In the present work, we aimed to elucidate the anti-aging effects of sesamin on several aging-related phenotypes in muscle, brain and midgut using *Drosophila* senescence-accelerated models ($Sod1^{n1}$ mutant and *Sod1*-depleted flies). We also determined the expression levels of several anti-oxidative genes and DNA repair genes using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). In addition, we analyzed the metabolism of sesamin in *Drosophila*.

Materials and Methods

Fly Stocks and Culture

Canton S was used as a wild-type stock. To examine the lifespan extension and anti-aging effects of sesamin, a *Drosophila* senescence-accelerated model, $Sod1^{n1}$ mutant was used[29]. This mutant shows a considerably declined SOD activity due to an aminoacid substitution, without the reduced mRNA level[29]. Our previous work revealed that this fly model displayed several aging phenotypes faster than wild-type flies[22]. We used this homozygous mutant for a lifespan assay and climbing assay as described in the following sections. For examining aging phenotypes appeared in certain adult tissues, we performed depletion of *Sod1* gene at the adult stage. We used the Gal4/UAS system, ectopic gene expression system in *Drosophila* for this purpose[30,31]. For inducing dsRNA against the *Sod1* mRNA, $UAS\text{-}Sod1RNAi^{F103}$ stock was used[22]. For depletion in muscle, *Mef2-Gal4. R3 (Mef-Gal4)* was used as a Gal4 driver stock. For depletion in adult brains, *Actin5C-Gal4* was used. We performed ubiquitous depletion of the *Sod1* gene at the adult stage and observed the aging-related phenotypes in adult brains. Gal4 driver stocks (*Actin5C-Gal4* and *Mef2-Gal4*) were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). We designated $Mef2\text{-}Gal4>UAS\text{-}Sod1^{F103}$ flies (muscle-specific *Sod1* depletion) and $Actin5C\text{-}Gal4>UAS\text{-}Sod1^{F103}$ flies (ubiquitous *Sod1* depletion) thereafter. To visualize intestine stem cells (ISCs) and their daughter cells, known as enteroblasts (EBs), in adult midguts, a stock carrying *esg-GAL4* and *UAS-GFP* (*esg>GFP*), provided by the *Drosophila* Genetic Resource Center (Kyoto, Japan), was used[32]. *P{GstD1-GFP}* (*GstD-GFP*) was used as a marker to visualize ROS accumulation[22,33]. All fly stocks were maintained on a normal cornmeal diet at 25°C. Every 1 L of normal cornmeal food included 7.2 g agar, 100 g glucose, 40 g dried yeast, and 40 g cornmeal. All ingredients were mixed well and boiled for 10 min. After cooling to 70°C, 5 mL of 10% parahydroxybenzonate dissolved in ethanol and 5 mL propionic acid were added.

Lifespan Assay

The lifespan extension effect of sesamin was confirmed using our *Drosophila* senescence-accelerated model, $Sod1^{n1}$ mutants. The lifespan assay was performed as previously described[22,34]. To avoid differences in lifespan between mated and virgin females and to avoid the higher accidental mortality observed in females compared with males, male adults were selected for the lifespan assay. Young adults were collected within 24 h after eclosion. Twenty flies were reared in a

single plastic vial containing *Drosophila* instant medium (Formula 4-24, Blue, Wako, Osaka, Japan). Sesamin (Nacalai Tesque, Kyoto, Japan) was dissolved in dimethyl sulfoxide (DMSO), and the stock solution was diluted using distilled water to achieve final concentrations of 0.35 or 2 mg/mL in the diet. As controls, DMSO (1% and 2%, respectively) alone was added to the instant food at the corresponding concentrations. The assay was repeated three to six times (using a total of 76-97 $Sod1^{n1}$ mutant flies). Dead adults in each vial were scored every 12 h for $Sod1^{n1}$ mutants. Food vials were changed every 3 days. Survival curves were analyzed by the Kaplan-Meier test.

Climbing Assays

Climbing assays, that utilized a fly's instinct of negative geotaxis, were performed according to a previous protocol[22] with slight modifications. Briefly, newly eclosed $Sod1^{n1}$ mutant adults were collected and raised on a diet containing 2-mg/mL sesamin or 2% DMSO alone. Twenty adult flies of the same age (0, 5, 8, or 10 days after eclosion) were gently tapped with a constant strength to the bottom of a plastic culture tube (100 mm in height and 15 mm in diameter), and the number of flies that climbed up to a marked point (50 mm in height, half the height from the bottom of the tube) after 6 s were counted and scored as follows: 10 points for climbing over the point, 5 points for climbing up between the line and the bottom of the tube, and 0 points for staying at the bottom of the tube throughout. The average scores for each fly were calculated, and the mean scores of five repeated trials (a total of 100-200 flies) were presented[22,35].

Absorption and Metabolism

Five to seven-day-old wild-type flies were collected and fed a diet supplemented with 2 mg/mL sesamin dissolved in 2% DMSO or 2% DMSO alone for 7 days. The flies were then maintained on a diet without sesamin for 30 min to drain the undigested diet from the gut. The wings and legs were removed from the flies before homogenizing them to prevent incorporating any sesamin that may have adhered to the body surface. After briefly rinsing with Phosphate-Buffered Saline supplemented with 0.1% Triton X-100 (PBST), the flies were flash-frozen in liquid nitrogen. Frozen *Drosophila* adults were suspended in 200 μL of 0.1 M KCl and homogenized using a TissueLyser (QIAGEN Sciences Inc., Germantown, MD, USA) for 60 s. Sesamin and its metabolites were extracted from *Drosophila* homogenates using the method described by Bligh-Dyer[36]. Extracts were analyzed according to the method reported by Tomimori et al[37] using an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) coupled to a Quattro micro MS (Mass Spectrometry) system (IET; Mundelein, IL, USA). UV detection was performed at 280 nm. The limit of detection was 0.05 μg/mL in both sesamin and SC1. The MS spectrometer was operated in full scan and multiple reaction monitoring modes.

Immunostaining Procedures

For muscle immunohistochemistry, we collected newly eclosed flies with a muscle-specific *Sod1* depletion and raised them for 5, 20 or 30 days on diets supplemented with 2 mg/mL sesamin or 2% DMSO. For dissection of the indirect flight muscles, the thoraces of the adult flies were cut along a longitudinal midline in relaxing buffer (0.1 M KCl, 20 mM Tris-HCl, pH 7.2, 1 mM $MgCl_2$, and 1 mM EDTA), as described[22]. After removal of cuticles, a thin block of the thorax, containing the dorsal longitudinal muscles in indirect flight muscle, was dissected from the abdomen. The muscle samples were fixed in 4% paraformaldehyde for 30 min washed with PBST, and blocked with 10% normal goat serum. To identify abnormal protein aggregates, a mouse antibody reactive to both mono- and polyubiquitinated proteins (Enzo Life Sciences, Farmingdale, NY, USA; 1:300) was used. The samples were incubated with primary antibody overnight at 4°C. After washing with PBST, samples were incubated with Alexa Fluor 488-conjugated secondary antibodies. For simultaneous detection of myofibrils, we used rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA; 1:80) at room temperature and washed the samples with PBST. One image per fly was observed, and the pixels in one optic field (4.0×10^{-2} mm^2) were measured using a confocal microscope.

To visualize the DA neurons by immunohistochemistry in the brains of adult flies, whole brains were dissected from the flies with a ubiquitous depletion of *Sod1* on day 5, as described previously[22]. To collect an intact whole adult brain, the head capsule was carefully removed using one pair of fine forceps to avoid damage to the brain and optic lobes. The isolated brains were then fixed in 3.7% formaldehyde for 15 min and incubated with anti-tyrosine hydroxylase primary antibody (Millipore, Billerica, MA, USA) overnight at 4°C. After washing, samples were incubated

with Alexa Fluor 594-conjugated secondary antibodies. The number of visualized DA neurons in the PLL1, PPM1/2, PPM3, PPL2 and VUM clusters in the adult brains were counted.

To evaluate the aging phenotypes in the adult midgut, the accumulation of DNA damage and hyperproliferation of ISCs and EBs, which occur to regenerate the damaged epithelial cells, was assessed[28,38]. Anti-γH2AvD immunostaining was performed to detect DNA damage foci in ISCs and EBs that were recognized by the *esg>GFP* reporter. To simplify the experiments, flies carrying the wild-type allele of *Sod1* were subjected to extrinsic oxidative stress by feeding them paraquat, an agent known to induce free radical production, instead of utilizing the $Sod1^{n1}$ mutants. In previous studies[32], metformin was used as an anti-aging drug that suppressed DNA damage accumulation and, consequently, stem cell hyperproliferation in *Drosophila* adult midguts. Therefore, metformin was compared with sesamin. For midgut immunohistochemistry, newly eclosed *esg>GFP* flies were collected, aged on a standard fly food for 33 days, divided into three groups and fed instant medium supplemented with 1.5 mg/mL metformin, 2 mg/mL sesamin, or 2% DMSO alone for 7 days at 28°C. Flies were transferred to new food every 2 days. For the paraquat-feeding assay, 40-day-old flies were placed in empty vials in 10 mM paraquat (methyl viologen; Sigma-Aldrich, St. Louis, MO, USA) with 5% sucrose. For immunohistochemical analysis of the digestive tract, we prepared the midguts from adult flies as described previously[34]. After incubating for 18-20 h, the abdominal ventral epidermis of the flies was cut along the midline using a micro scissor. The epidermis was consequently opened in PBS by a pair of fine forceps to collect the internal digestive organs. The midgut was carefully isolated from the organs by removing the foregut, hindgut and malpighian tubes to avoid damage to a tubular structure of the gut. The isolated midguts were fixed at room temperature for 30 min in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA). After washing with PBST, samples were blocked with 10% normal goat serum and incubated overnight with the primary antibody rabbit anti-γH2AvD (1:300; Rockland Immunochemical Inc., Gilbertsville, PA, USA) at 4°C. After extensive washing with PBST, samples were incubated with Alexa Fluor 594-conjugated secondary antibodies, washed with PBST and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). For a quantitative analysis of cell numbers in the adult midgut, the number of DAPI-positive (total cells), esg-positive cells (corresponding to ISCs and EBs), and γH2AvD-positive cells in an area of the posterior midgut were counted using Image J software (NIH, Bethesda, MD, USA). The results were presented as a percentage of the number of γH2AvD-positive cells among esg-positive cells or esg-positive cells among total cells.

All immunostained samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed using an Olympus laser scanning confocal microscope (Fv10i, Olympus, Tokyo, Japan). Brightness and contrast of entire images were adjusted using Fv10i software. Image analysis was performed using Photoshop and Image J (NIH, Bethesda, MD, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction Analysis (qRT-PCR)

Quantitative Reverse Transcription Polymerase Chain Reaction Analysis (qRT-PCR) was performed to quantitate mRNA levels of four genes encoding antioxidative enzymes (*catalase, Sod1, Sod2, and Jafrac1/Thioredoxin peroxidase 1*), two genes encoding DNA repair enzymes (*Gadd45* and *CG9272*), and the *GstD1* gene. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from newly eclosed (within 24 h) $Sod1^{n1}$ mutant adults fed with instant medium with or without 2-mg/mL sesamin for 7 days. cDNA was synthesized from total RNA using a PrimeScript II Fidelity RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan) and an oligo dT primer. Real Time-PCR was performed using the FastStart Essential DNA Green Master (Roche, Basel, Switzerland) and a Light Cycler Nano instrument (Roche, Basel, Switzerland). qPCR primers were as follows: RP49-FW, 5'-TTCCTGGTGCACAACGTG-3', RP49-RV, 5'-TCTCCTTGCGCTTCTTGG3', α-tubulin84B-FW, 5'-GCTTCCTCATCTTCCACTCG-3', α -tubulin84B-RV, 5'-GCTTGGACTTCTTGCCGTAG-3', Catalase-FW, 5'-TTTCTCCTGGTGCCATTACC-3', Catalase-RV, 5'-CTGCAGCAGGATAGGTCCTC-3', SOD1-FW, 5'-AGGTCAACATCACCGACTCC-3', SOD1-RV, 5'-GTTGACTTGCTCAGCTCGTG-3', SOD2-FW, 5'-TGGCCACATCAACCACAC-3', SOD2-RV, 5'-TTCCACTGCGACTCGATG-3', Gadd45-FW, 5'-GATCCCTCTTCTGCCTGATG-3', Gadd45-RV, 5'-TCGTCCACCTTGATCACGTA-3', Jafrac1-FW, 5'-ACCGAGATCATTGCGTTCTC-3', Jafrac1-RV, 5'-AAGTGGGTGAACTGGCTGTC-3', GstD1-FW, 5'-AAGATCAATCCC-

CAGCACAC-3', GstD1-RV, 5'-GGTCTTGCC-GTACTTCTCCA-3', CG9272-FW, 5'-GGTTCG-GCTACTGTGGTCAT-3', CG9272-RV, 5'-TATT-TGGGGTTCCACCTCCT-3'. All qRT-PCR experiments were performed in triplicate, and the results were taken as the average of three replicates in each group. The ΔΔCt method was used to determine the differences in target gene expression relative to the reference *Rp49* gene expression.

Evaluation of Oxidative Stress

The newly eclosed adults ubiquitously depleted for *Sod1 (Actin5C>Sod1RNAi)* were respectively raised on a diet supplemented with or without 2 mg/mL sesamin for 5 and 10 days. The *GstD1* gene is used as a marker for estimating the extent of ROS accumulation because its expression is induced in response to the accumulation of oxidative stress.

Statistical Analysis

A survival curve was calculated following Kaplan-Meier survival estimation and analyzed by log-rank test between the sesamin-treated group and the appropriate control group. For the comparisons of the two groups we used the Student's *t-test.* One-way ANOVA with post-hoc Tukey's test was applied to assess the differences in more than two groups. Two-way ANOVA with post-hoc Student's *t*-test was performed to compare the mean differences between groups that were split into two independent variables. Statistical analyses were performed using IBM SPSS statistics 25 software (IBM, Armonk, NY, USA). Data were considered significant at p-values < 0.05.

Results

Sesamin Feeding Extended the Lifespan of Drosophila Adults

As continuous administration of sesamin to wild-type *Drosophila* adults extended their life span in a previous study (see introduction), we first confirmed the lifespan extension effect of sesamin using our *Drosophila* senescence-accelerated model, $Sod1^{n1}$ mutants, in which ROS accumulation was amplified. $Sod1^{n1}$ mutant adults displayed a shortened adult lifespan, and the mean time required for 50% lethality in the presence of 1% and 2% DMSO was 7.1 and 6.6 days, respectively, and the longest adult lifespans were 12.5 and 12.5 days on average, respectively (Figure 1). The lifespan of *Drosophila* adults fed with 2 mg/mL sesamin was significantly extended compared with that of flies fed with 2% DMSO ($p < 0.001$), and the mean time required for 50% lethality was 11.6 days in the former. Feeding of 0.35 mg/mL sesamin also extended the lifespan compared to

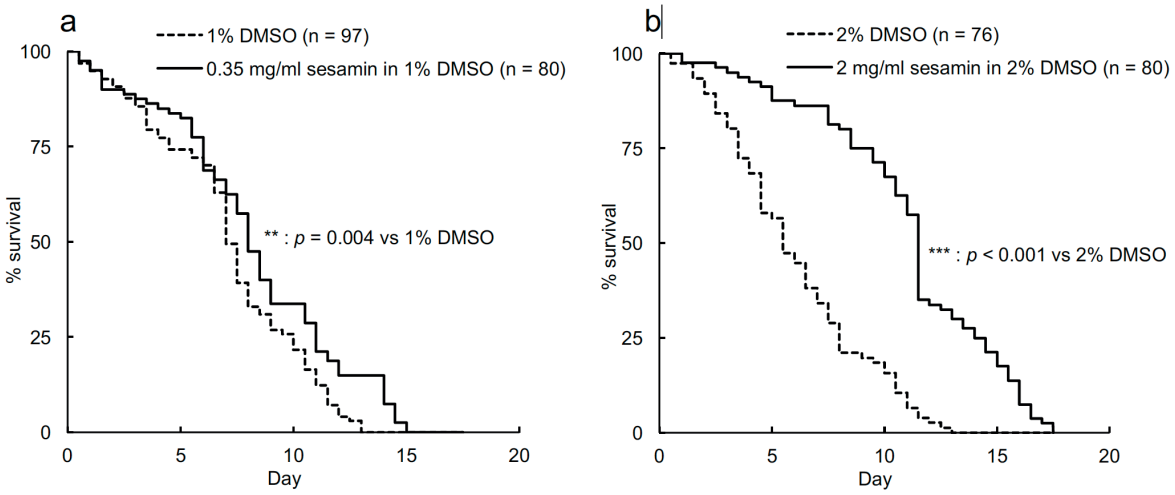


Figure 1. Sesamin extends lifespan in the *Drosophila* senescence-accelerated model, $Sod1^{n1}$ mutant adult flies. Lifespan curves in adult flies fed with or without 0.35 mg/mL sesamin in 1% DMSO (n = 80 and 97, respectively), and with and without 2 mg/mL sesamin in 2% DMSO (n = 80 and 76, respectively). The results represent the average of four repeated experiments. Curves were plotted using Kaplan-Meier survival analysis. A log-rank test was performed for each pair of adults fed with the control diet and adults fed with the sesamin diet. Note that a significant lifespan extension was observed in both cases, when flies were fed with 0.35 mg/mL (p = 0.004, log-rank test) and 2 mg/mL sesamin ($p < 0.001$, log-rank test).

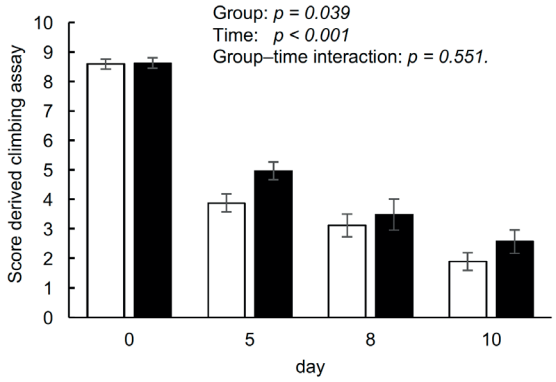


Figure 2. *Drosophila* adults fed with sesamin retain a higher climbing activity than control diet-fed adults. The locomotor activity of the *Drosophila* senescence-accelerated model, $Sod1^{n1}$ mutant flies, fed with a diet supplemented with 2% DMSO (white bars) or 2 mg/mL sesamin dissolved in 2% DMSO (black bars) was examined at 0, 5, 8, and 10 days after eclosion using a climbing assay. The y-axis shows the scores for flies that climbed above a line drawn at a certain height from the bottom, within a predetermined period (see Materials and Methods), (n = 100-200 flies from five repeated assays). Data are shown as mean ± SE. There was no difference in the group-time interaction ($p = 0.551$).

feeding with 1% DMSO ($p = 0.004$). Feeding of 2 mg/mL sesamin was more effective in extending adult lifespan than feeding with other sesamin concentrations; therefore, we used this final concentration for further examination of the anti-aging effects of sesamin.

Age-Dependent Loss of Locomotor Activity was Suppressed in Drosophila Adults Fed with Sesamin

We examined whether the lifespan extension in adults fed with sesamin was a consequence of aging suppression. We performed a climbing assay to quantitate adult locomotor activity (Figure 2). The score derived from the climbing assay for control $Sod1^{n1}$ mutant flies decreased with aging (Figure 2). However, feeding with 2 mg/mL sesamin significantly improved this decline of locomotor activities with aging ($p = 0.039$). These results indicate that age-dependent impairment of locomotor activity was suppressed in *Drosophila* adults fed with sesamin.

Sesamin Suppressed the Age-Dependent Accumulation of Abnormal Protein Aggregates in Indirect Flight Muscles

As we showed that sesamin can suppress the aging-related decrease in locomotor activities, we examined whether sesamin suppressed the accumulation of abnormal protein aggregates in adult indirect flight muscles. As flies age, protein aggregates containing damaged proteins accumulate in their muscles. We performed immunostaining in the indirect flight muscles of a muscle-specific *Sod1* depletion (*Mef2>Sod1RNAi*). Representative confocal microscopy images showing polyubiquitinated protein (Figure 3a-f) and merged images with myofibrils (Figure 3a′-f′), and presented the average data for polyubiquitinated protein (Figure 3g). We showed an increase in protein aggregates with aging in the indirect flight muscles of 5-, 20-, and 30-day-old flies fed with the control diet (62, 333, and 459 pixels, respectively). In contrast, we found a significant decrease in the number and size of aggregates in muscles from 5-, 20-, and 30-day-old flies fed with the sesamin diet ($p < 0.001$ for 47 pixels; $p < 0.001$ for 218 pixels, and $p < 0.001$ for 276 pixels, respectively) (Figure 3g). These observations indicated that the sesamin diet markedly suppressed the accumulation of protein aggregates that increased with aging.

Expression of GstD1 (a Marker Gene for Oxidative Stress) was Partially Suppressed in Drosophila Adults Fed with Sesamin

As ROS accumulation is associated with the progression of aging, we examined whether the sesamin diet influenced ROS accumulation. As ubiquitous *Sod1*-depleted flies fed with the DMSO diet aged, we observed more intense GFP fluorescence along their whole body, especially in the abdomens and thoraxes (Supplementary Figure 1a-c). In contrast, ubiquitous *Sod1*-depleted flies fed with the sesamin diet displayed less intense GFP signals (Supplementary Figure 1d, e) in 5- and 10-day-old flies. Thus, induction of the oxidative stress marker gene, *GstD1*, was partially suppressed in *Drosophila* adults fed with sesamin.

The Sesamin Diet Partially Suppressed the Age/Oxidative Stress-Dependent Loss of DA Neurons in Drosophila Adults

To further understand the mechanisms underlying the role of sesamin in alleviating the age-dependent loss of locomotor activity, we examined whether it could also suppress the loss of DA neurons in *Drosophila* adult brains harboring ROS accumulation. We confirmed that the loss of DA neurons occurred in each of the five typical clusters in adult brains prepared from 5-day-old control ubiquitous *Sod1*-depleted adults (Figure

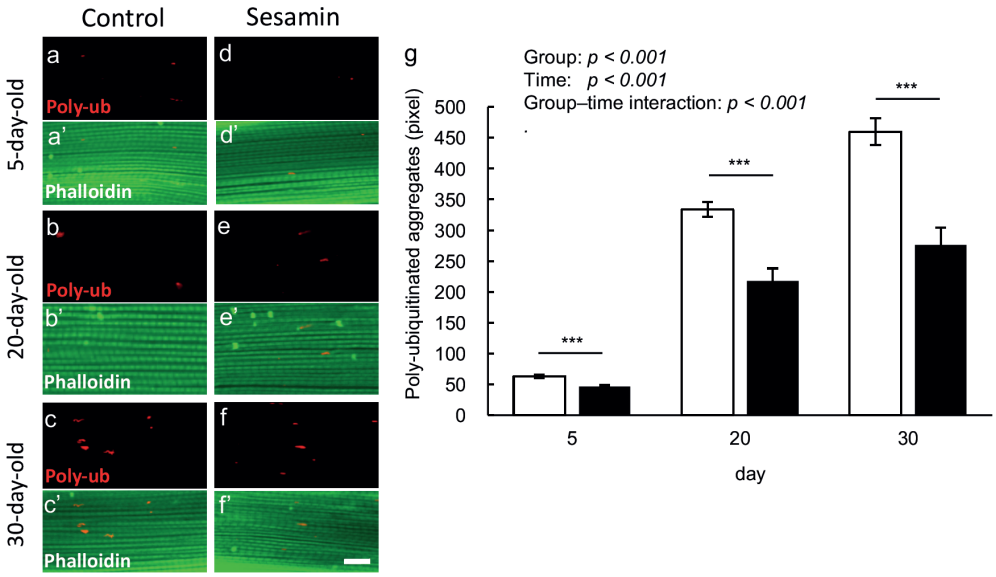


Figure 3. Sesamin suppresses the accumulation of polyubiquitinated protein aggregates in the myofibrils of the indirect flight muscles. Immunostaining of the indirect flight muscles dissected from flies with a muscle-specific depletion of *Sod1 (mef2<Sod1RNAi^{F103})* using an anti-ubiquitin-conjugated antibody (red) ***a-f***, and phalloidin staining for F-actin (green) ***a'-f'***. Adult flies were collected within 24 h after eclosion, aged for 5 days ***a, d,*** 20 days ***b, e,*** and 30 days ***c, f,*** on a diet supplemented with 2% DMSO alone ***a-c,*** or 2 mg/mL sesamin dissolved in 2% DMSO ***d-f***. Scale bar represents 10 μm ***g,*** The average pixel numbers of the sum of protein aggregates containing polyubiquitinated proteins per single confocal optic fields (4.0 × 10-2 mm2) in control (white bars) and sesamin (black bars) fed flies are shown on the y-axis. Data are shown as mean ± SE. At least 10 images of muscle samples prepared from at least 10 flies were observed. Two-way ANOVA with post-hoc Student's *t*-test were applied to compare the mean differences. ***$p < 0.001$ (Student's *t*-test).

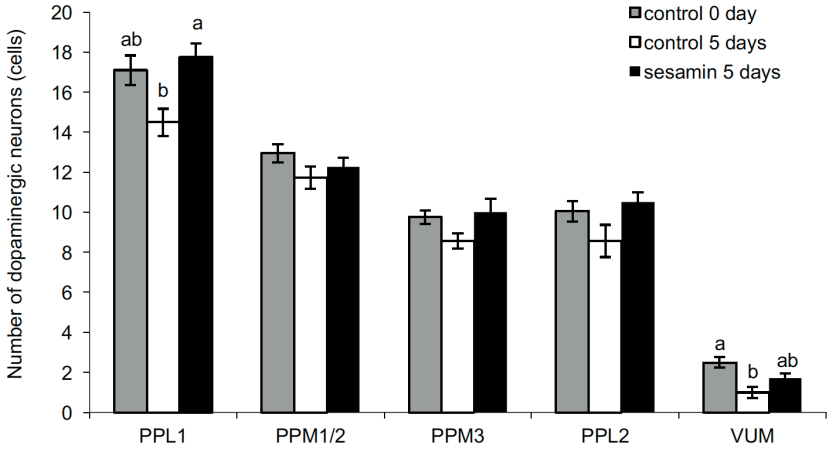


Figure 4. Suppression of the oxidative stress-dependent loss of DA neurons in the brains of *Sod1^{n1}* mutant adult flies fed with sesamin. Adult whole brains were dissected from 0- and 5-day-old *Sod1^{n1}* mutant flies that were collected within 24 h after eclosion and raised on a diet supplemented with either 2% DMSO (control) or 2 mg/mL sesamin dissolved in 2% DMSO (sesamin). Immunostaining of the brains using an anti-tyrosine hydroxylase antibody to visualize DA neurons was performed and observed using a confocal microscope. The number of DA neurons in each of the five main neuronal clusters (PPL1, PPM1/2, PPM3, PPL2 and VUM) was counted and is summarized as a bar graph. Data are shown as mean ± SE. Statistical analysis in each cluster was performed by one-way ANOVA with post-hoc Tukey's test. Different letters indicate significant differences between groups ($p < 0.05$). There were no differences in PPM1/2, PPM3, and PPL2 clusters by one-way ANOVA.

4). Sesamin feeding suppressed the decline of DA neurons to the same levels as the 0-day-old flies. In particular, PPL1, the largest DA neurons cluster in the brain, was significantly retained in flies fed with sesamin compared with that in flies fed with the control diet ($p < 0.05$; Figure 4).

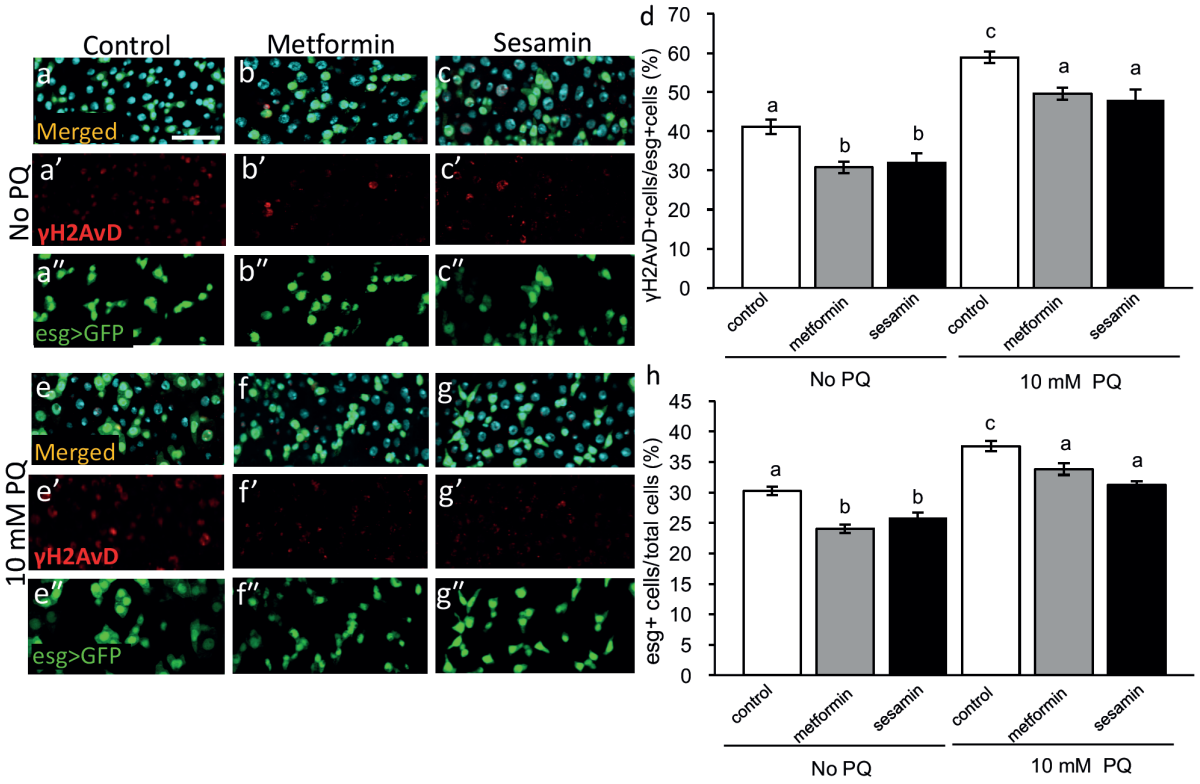


Figure 5. Sesamin inhibits DNA damage accumulation and hyperproliferation of ISCs and EBs in intestinal epithelia in aged flies fed with or without paraquat. Representative confocal microscopy images show DNA damage accumulation in ISCs and EBs without ***a'-c',*** and with 10 mM paraquat ***5e'-g',*** and hyperproliferation of ISCs and EB cells without ***5a''-c'',*** and with 10 mM paraquat ***5e''-g'',*** in the midgut of aged adults (40 days old). ISCs and their daughter EBs were visualized using *esg>GFP* reporter (*green*) and DAPI (blue). Scale bar represents 10 μm ***d.*** The average percentages of cells showing γH2AvD immunostaining signals in their nuclei among the ISCs and EBs, labelled as esg^{+} cells, observed per single optic field using confocal microscopy are shown on the y-axis. At least 35 confocal images of midgut samples prepared from at least seven flies were observed for each condition. ***H,*** The average percentages of ISCs and EBs among the total number of intestinal cells observed per single optic field in confocal microscope are shown on the y-axis. 25 images of the midgut samples prepared from at least five flies were observed in each condition. Data are shown as mean ± SE. Statistical analyses were performed using a one-way ANOVA with post-hoc Tukey's test. Different letters indicate significant differences between groups ($p < 0.05$).

Sesamin Suppressed DNA Damage Accumulation and Hyperproliferation of Intestine Stem Cells in the Adult Midgut

As flies age, intestinal stem cells (ISC) proliferate more frequently to compensate for damaged epithelial cells in the adult midgut[27-29,32], we examined whether sesamin and metformin showed anti-aging effects in the midguts from adults carrying $Sod1^{+}$ allele. Representative confocal microscopy images indicating DNA damage accumulation (Figure 5a′-c′, 6e′-g′), IPC hyperproliferation (Figure 5a″-c ″, e″ -g″), and the average data of each (Figure 6d, h) are shown. Metformin was used as a drug having the anti-aging effect that suppresses aging phenotype in the adult midgut, although its effects on other aging phenotypes have not been examined. Metformin feeding decreased the frequency of cells exhibiting nuclear DNA damage foci from 41% to 31% ($p < 0.05$; Figure 5a′, b′, d) and from 58% to 49% under extrinsic oxidative stress conditions ($p < 0.05$; Figure 5e′, f′, d). Metformin feeding also decreased the frequency of hyperproliferation of the total intestinal epithelial cells from 30% to 24% ($p < 0.05$; Figure 5a″, b″, h) and from 37% to 35% under extrinsic oxidative stress conditions ($p < 0.05$; Figure 6e″, f″, h). The sesamin diet also decreased the frequency of nuclei exhibiting DNA damage foci to 31% ($p < 0.05$; Figure 5a′, c′, d) and 48% under extrinsic oxidative stress conditions ($p < 0.05$; Figure 5e′, g′, d). Moreover, sesamin feeding significantly decreased the hyperproliferation to 26% ($p < 0.05$; Figure 5a″, c″, h) and 31% under oxidative stress conditions ($p < 0.05$; Figure 5e″,

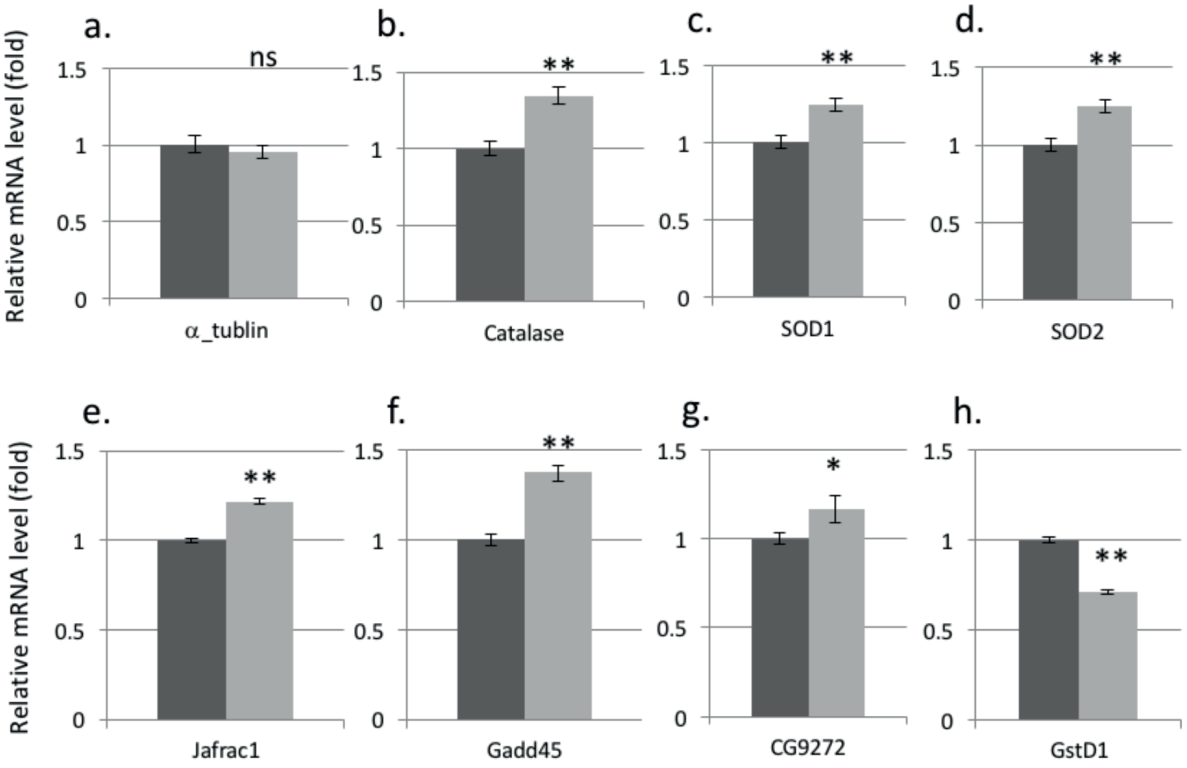


Figure 6. Quantitation of mRNA of seven anti-oxidative response-related genes in the *Drosophila* senescence-accelerated model, *Sod1*^{n1} mutant adults fed with or without sesamin. Relative mRNA levels of each gene in adults fed with a diet containing sesamin (*light gray bars*) compared with those in adults fed with a diet without sesamin (*control, dark gray bars*) were calculated using Real Time-qRT-PCR analysis. Total RNA was isolated from adults fed with a diet containing 2 mg/mL sesamin and from those fed with a diet containing 2% DMSO as a control for 7 days. ***A,*** α-tubulin was used as a housekeeping gene, ***b-d, f,*** genes for antioxidative enzymes: *catalase* ***b,*** *Sod1* ***c,*** *Sod2* ***d,*** and *Jafrac1* ***e***. DNA repair genes, *Gadd45* ***f,*** and *CG9272* ***g. H,*** *GstD1*, expression of which is induced in response to oxidative stress. The qRT-PCR analysis to quantitate the amount of mRNA of each target gene was repeated three times. Statistical analysis was performed using Student's *t-test*. ns, not significant; *$p < 0.05$; **$p < 0.01$.

g″, h). These results indicate that sesamin can remarkably suppress DNA damage accumulation and the IPC hyperproliferation with aging, and the effects were comparable to those of metformin.

Six Genes Encoding Enzymes that Remove Oxidative Stress were Upregulated in Drosophila Adults Fed with Sesamin

The mRNA levels of four antioxidative enzymes (*catalase, SOD1, SOD2, and Jafrac1*) increased by 20%-30% in *Sod1*^{n1} mutant flies fed with sesamin compared with those in adults fed with the control diet ($p < 0.01$; Figure 6). The mRNA levels of the genes encoding two DNA repair factors (*Gadd45* and *CG9272*) also increased by 20%-30% in adult flies fed with sesamin. In contrast, *GstD1* expression decreased by 30%. This result is consistent with the observation that the induction of *GstD1-GFP* was suppressed by the sesamin diet (Supplementary Figure 1). No significant differences were observed in levels of the housekeeping gene α-tubulin.

Sesamin was Absorbed and Metabolized in Drosophila Adults

We confirmed whether sesamin was absorbed and metabolized in the wild-type *Drosophila* adult body. HPLC chromatograms of the *Drosophila* extracts are shown in Figure 7. Sesamin (black arrow) and its metabolite peak (indicated by Peak 1 arrow) were detected in extracts from adults fed with sesamin. MS in the negative ion mode for peak 1 showed a deprotonated molecular ion of m/z 341 [M-H]-. Furthermore, the retention time of peak 1 was identical to that of the authentic SC1 standard with multiple reaction monito-

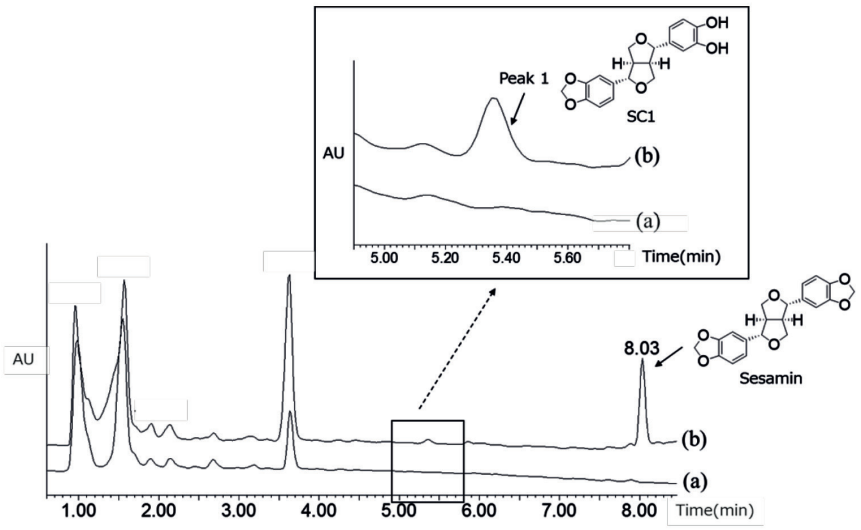
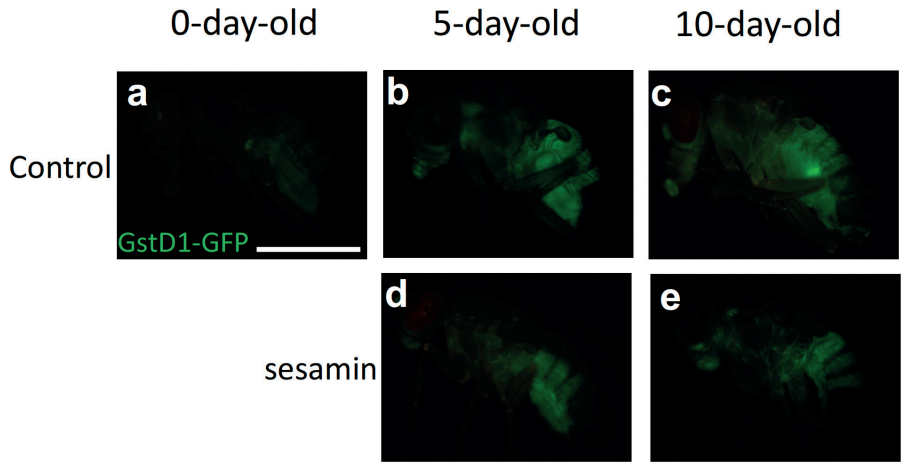


Figure 7. HPLC chromatogram of *Drosophila* extracts. The HPLC chromatogram of extract of wild-type adults fed with ***a,*** control diet supplemented with 2% DMSO and ***b,*** diet supplemented with 2 mg/mL sesamin dissolved in 2% DMSO for 7 days.

ring at m/z 341.2 > 176.2. These data suggest that sesamin was absorbed and metabolized to SC1 in *Drosophila* adults, as observed in mammals.

Discussion

Sesamin not only promotes lifespan extension in *Drosophila* adults[16] but also suppresses several types of aging phenotypes, such as age-dependent accumulation of aggregates containing damaged proteins in the muscle and loss of DA neurons in adult brains. We also showed that a sesamin diet suppressed stem cell hyperproliferation, a consequence of DNA damage accumulation in intestinal epithelial cells. These results in *Drosophila* are consistent with previous findings showing that sesamin has suppressive effects that restore tissue



Supplementary Figure 1. Suppression of *GstD1* expression correlates with ROS accu-mulation in sesamin-fed *Drosophila* adult flies. Stereofluorescence microscopy images of *Drosophila* adult (*lateral view of flies*). Induction of *GstD1* expression was monitored using a GFP reporter gene under the control of the *GstD1* enhancer, a marker for ROS accumulation. To avoid collecting autofluorescence from ovaries, we selected male flies carrying a *GstD-GFP* reporter. Flies with a ubiquitous *Sod1* depletion (*Actin5C>Sod1RNA*i, *GstD-GF*P) (adults with a *Sod1* depletion) were reared at 28°C for 0 days a, 5 days ***b, d***, and 10 days c, e, after eclosion with 2% DMSO ***b, c,*** or on 2-mg/mL sesamin dissolved in 2% DMSO ***d, e***. A distinctive reduction in the intensity of GFP fluorescence was observed in adults fed with sesamin, compared with that in the control adults. Scale bars represent 250 μm.

dysfunction or damage in several animal disease models. For example, sesamin inhibited the decline in exercise capacity and mitochondrial function impairment in the skeletal muscle of mice with high-fat diet-induced diabetes[14]. Furthermore, it displayed a hepatoprotective effect against oxidative stress and apoptosis in mice exposed to CCl_4[39]. In addition, it also suppressed behavioral dysfunctions and exhibited neuroprotective effects in chemically induced Parkinson's disease rat and mouse models via inhibition of oxidative stress and apoptosis in neuronal cells[40,41]. However, these effects of sesamin in animal models do not necessarily indicate its potential anti-aging effects in mammalian models and humans. Current studies on aging use a variety of model organisms from yeast and worms to mice. However, it is not easy to extrapolate the results derived from yeast and worms to higher organisms. Nonetheless, aging studies using mice are time-consuming and cost-intensive. On the other hand, *Drosophila* provides a useful tool to advance studies on aging and evaluate chemicals and natural products with anti-aging activities quickly and efficiently[18,19]. The *Drosophila* senescence-accelerated models used in this study enable a detailed observation of several aging phenotypes in only 2 weeks[22]. As our previous study indicated that *Sod1*-depleted adults showed extra ROS accumulation and that the flies displayed several aging phenotypes at an earlier adult stage, we believed that the aging-accelerated phenotypes, which appeared in $Sod1^{n1}$ mutant having a lower *Sod1* activity are associated with ROS accumulation. The *Drosophila* results represent the first evidence regarding the anti-aging effects of sesamin in animal muscle, brain and intestine.

We showed that two chemical compounds, metformin and sesamin, have anti-aging effects that delayed the progression of aging phenotypes in *Drosophila* adult guts. A previous study[32] has reported that metformin suppresses the characteristic features of aged cells in the *Drosophila* midgut, such as DNA damage accumulation and stem cell hyperproliferation. After we established a reliable experimental system for monitoring the anti-aging effects, we investigated whether sesamin delayed the progression of aging phenotypes of *Drosophila* midgut. Sesamin also inhibited age- and oxidative stress-inducible DNA damage and the hyperproliferation of intestine stem cells. The present work demonstrated that sesamin is a potential anti-aging drug as well as metformin[32]. To further confirm the finding, it is also fascinating to examine whether sesamin can show a suppression effect on tissue damage by bacterial liposaccharide-induced ROS[42]. In contrast to published data demonstrating that metformin activates AMP-activated kinase (AMPK)[43], no study has shown the biochemical data suggesting that sesamin can activate AMPK. Therefore, it is reasonable to speculate that an entirely different mechanism is responsible for the anti-aging effects of sesamin. A previous DNA microarray study demonstrated that sesamin affected the transcription of various genes, including those encoding antioxidative enzymes in a rat model[44]. In addition, sesamin feeding reportedly induced the antioxidative enzyme activities such as superoxide dismutase, glutathione S-transferase, and glutathione peroxidase in the liver of mice models[2,45]. A recent *Drosophila* study[16] reported that sesamin influenced the expression of antioxidant genes, such as *Sod1, Sod2, catalase,* and *Rpn11.* Our qRT-PCR analysis also confirmed that the sesamin diet resulted in the simultaneous upregulation of antioxidative genes, including *Sod1, Sod2,* and *catalase*, as well as of DNA repair genes. Consistent with our observations, dietary sesamin suppressed ROS accumulation in the fly body. A possible transcription factor involved in the oxidative stress response is Nrf2/Cnc-Keap1[33]. Hamada et al[13] showed that an SC1, anti-oxidative catechol metabolite, enhanced Nrf2 activation in cultured PC12 cells. In this work, sesamin metabolite, SC1, was detected in the adult bodies fed with sesamin. Therefore, sesamin feeding could induce the antioxidative enzyme activities via induction of Nrf2/keap1 system in *Drosophila* adults. Another possible transcription factor involved in the oxidative stress response is Foxo transcription factor[46-49]. To further understand the molecular mechanisms underlying the anti-aging effects of sesamin, it is necessary to investigate whether sesamin feeding can activate Nrf2 or Foxo in the *Drosophila* model.

Sesamin was reportedly metabolized into SC1 in mammalian bodies by a cytochrome P450 enzyme[12]. Similarly, 90 P450-related genes including pseudogenes have been identified in *Drosophila* genome[50,51]. Although a *Drosophila* counterpart of the sesamin-metabolizing enzyme has not been characterized yet, a presence of SC1 in sesamin-feeding adults strongly suggests that the counterpart enzyme(s) function in *Drosophila* as shown in mammals. In this experiment, extracts from the whole body were used as HPLC samples for sesamin and its metabolite analysis, because

Drosophila is too small for analyzing sesamin and its metabolites from each organ. Therefore, sesamin which remained without being absorbed in the gastrointestinal tract and/or sesamin adhering to the body surface may have been detected, and it is difficult to compare sesamin with SC1 peak. Further studies are needed as to how efficiently the sesamin was metabolized in the body of *Drosophila*.

Our data on *Drosophila* indicate that sesamin possesses anti-aging effects that suppress age-dependent muscle impairment. A previous study[22] showed that the protein aggregates that accumulate in the muscle with aging are associated with malformed mitochondria, damaged by oxidative stress. Maharjan et al[52] demonstrated that sesamin can rejuvenate mitochondria damaged by oxidative stress in cultured cells. Takada et al[14] also reported that sesamin prevented the decline in exercise capacity via maintenance of mitochondrial function and attenuation of oxidative stress in skeletal muscle. Studies[10,14,15] using experimental animals have also demonstrated that sesamin feeding inhibited NADPH oxidase activity and the expression of its subunits, resulting in suppression of superoxide generation. Therefore, the anti-aging effects of sesamin in the muscle may involve inhibition of oxidative stress in the mitochondria by the suppression of superoxide generation. Another possibility is that this compound may stimulate the induction of autophagy to remove damaged mitochondria. A previous study[53] has reported that sesamin can induce autophagy in cancer cell lines. Although there is no direct evidence of a relationship between this compound and oxidative stress-induced autophagy, sesamin extends the lifespan of *C. elegans* by activating autophagy[54]. Furthermore, other studies[55,56] have shown that oxidative stress could decrease proteasomal activity *in vitro* and *in vivo*; therefore, it is possible that sesamin regulates protein homeostasis by inhibiting oxidative stress-mediated proteasomal impairment during aging.

It was previously reported[22] that apoptosis is involved in the age-dependent loss of DA neurons in *Drosophila* adult brains. Using the same experimental system, we showed that a sesamin diet partially suppressed the loss of DA neurons. A previous study[13,57] has reported that a metabolite of sesamin inhibits oxidative stress-induced cell death in PC12 cells. Our findings regarding the neurons in *Drosophila* adults fed with sesamin are consistent with this result suggesting that sesamin has an antioxidative effect on the neuronal precursor cell line. Therefore, it is possible that the antioxidative activity of sesamin underlies its suppressive effects regarding the age-dependent loss of neurons.

Conclusions

We evaluated the anti-aging effects of sesamin using the *Drosophila* senescence-accelerated models. Sesamin suppressed several aging-related phenotypes that appeared in muscle, brains and intestines of *Drosophila* adults. These observations are the first direct evidence of the anti-aging effects of sesamin at an individual level. Sesamin also upregulated the expression of several anti-oxidative and DNA repair genes. We surmise that sesamin exerts anti-aging effects in several tissues by inducing anti-oxidative and DNA repair genes, thus extending the lifespan of *Drosophila*.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgments

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Statement of Interests

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