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Grape Extract Protects Mitochondria from Oxidative Damage and Improves Locomotor Dysfunction and Extends Lifespan in a Drosophila Parkinson's Disease Model

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Abstract

A botanical extract (Regrapex-R®) prepared from whole grape (*Vitis vinifera*) and *Polygonum cuspidatum*, which contains polyphenols, including flavans, anthocyanins, emodin, and resveratrol, exhibited dose-dependent scavenging effects on reactive oxygen species (ROS). The extract inhibited increases of ROS and protein carbonyl in isolated rat liver mitochondria following exposure to 2,2′-azobis (2-amidino propane) dihydrocholoride (AAPH), a potent lipid oxidant generator. The antioxidant effects of this extract were further demonstrated by protecting enzyme activities of the mitochondrial respiratory electron transport chain (complexes I and II) and pyruvate dehydrogenase in isolated liver mitochondria with AAPH insult. In human neuroblastoma cells (SK-N-MC), pretreatment of extract protected cells against AAPH induced oxidation in maintaining cell viability and inhibiting excessive ROS generation. Extract was fed to transgenic *Drosophila* expressing human α-synuclein. This model for Parkinson disease recapitulates essential features of the disorder, including loss of dopaminergic neurons in the substantia nigra and a locomotor dysfunction that is displayed by a progressive loss of climbing ability measured using a geotaxis assay. Male transgenic flies fed the extract (0.16–0.64 mg/100 g of culture medium) showed a significant improvement in climbing ability compared to controls. Female transgenic flies showed a significant extension in average lifespan. These results suggest that Regrapex-R is a potent free radical scavenger, a mitochondrial protector, and a candidate for further studies to assess its ability to protect against neurodegenerative disease and potentially extend lifespan.

Introduction

The health benefits of consuming grapes (*Vitis vinifera*) and grape seed extract (GSE) have been widely reported, and products based on such extracts are available worldwide. In Europe and Asia, botanical extracts are often consumed as phytomedicines, whereas in the United States such products are marketed and sold as dietary supplements. Approximately 62% of U.S. adults are using some form of complementary and alternative medical approach for preventing and managing chronic disease and consumption of botanical extracts, comprising a large part of this practice; however, the health claims of such products remain largely unsubstantiated.[1]

The health benefits attributed to the polyphenols in grape extract are wide ranging and include evidence of cardioprotective effects,[2–4] including reducing the incidence of atherosclerosis[5,6] and protecting against hypertension in animal models[7,8]; anticancer effects including colon cancer,[9] prostate cancer,[10] and oral squamous carcinomas[11]; and neurodegenerative diseases like Alzheimer disease.[12,13] In the case of neurodegenerative disease, it has been suggested that GSE exerts its effects through changes in the levels of specific brain proteins that may be responsible for the neuroprotective effects observed.[14]

Extracts of grape and GSE are rich in polyphenols, including several bioactive chemical classes, primarily the proanthocyanidins, which are a mixture of procyanidin

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derivatives of the flavan-3-ol class of flavonoids. This class includes catechin and epicatechin as dimers and oligomers, including their gallic acid esters. GSE proanthocyanidins comprise approximately 60–70% of the polyphenol content of grapes.[15,16] The primary health benefits of grape extract are believed to be due to its well-documented and potent antioxidant properties, which provide excellent protection against oxidative stress and free radical-mediated tissue injury.[17] Grape extract has been shown to be highly bioavailable and provides significantly greater protection against free radicals and free radical-induced lipid peroxidation and DNA damage than vitamins C, E, and β-carotene.

However, the effects of grape extract on mitochondria and Parkinson disease (PD) have not been well studied. The present study evaluated the antioxidant effects of a well-characterized grape extract preparation (Regrapex-R®) using both in *vitro* and *in vivo* assays. The results demonstrated Regrapex-R enhanced rat liver mitochondrial enzyme complexes and ameliorated the locomotor dysfunction that were exhibited by the α-synuclein transgenic *Drosophila* model for PD, in addition to its free radical scavenging activity.

Materials and Methods

Chemicals

Tris base and nicotinamide adenine dinucleotide (NADH) were purchased from Amersco Inc. (Palm Harbor, FL); 2,6-dichloroindophenol indophenol (DCPIP) from Merck & Co. Inc. (USA); rotenone from Riedel De Haen Seelze (Hannover, Germany); 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN), coenzyme Q1, antimycine A, *p*-iodonitrotetrazolium violet (INT), dithiothreitol, and thiamin pyrophosphate from Sigma Chemical Co. (St. Louis, MO); and 2,2′-azobis (2-amidino propane) dihydrocholoride (AAPH) from Wako Pure Chemical Industries, Ltd. Dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR), and the BCA Protein Assay kit for protein quantification was from Pierce (Pierce Biotechnology, Inc., Milwaukee, WI). Other chemicals were all A.P. reagents from local vendors.

Grape extract

A grape extract fortified with resveratrol commercially available as a dietary supplement was obtained from Biophysica Inc. (San Diego, CA).[18] Regrapex-R, manufactured by Interpharma Praha (Praha 12–Modrany, Czech Republic), contains a whole-grape (*Vitis vinifera*) extract enriched with purified powdered extract from dried roots of *Polygonum cuspidatum* (1 gram of bulk consists of 800 mg of whole grape extract and 200 mg of dried root powder from *P. cuspidatum*). One gram of Regrapex-R contains 100 mg of resveratrol complex (*trans*-resveratrol and its glycosides), 10 mg of emodin complex (emodin and emodin glycosides), 450 mg of polyphenols, and 12 mg of anthocyanins.

Grape extract solution preparation

A stock solution was prepared with water for electron spin resonance (ESR) assay of hydroxyl radical or with dimethylsulfoxide (DMSO) for other assays.

Free radical scavenging

Hydroxyl radical, superoxide radical, and lipid radicals were detected using spin trapping agents with an ESR spectrometer.[19–21] Briefly, hydroxyl radical was detected in a 50-μL reaction mixture containing 100 mM DMPO, 100 mM hydrogen peroxide (H_2O_2), 0.3 mM Fe (II)/0.15 mM diethylene triamine pentaacetic acid (DTPA), and 30 μL of sample. The reaction was initiated by 0.3 mM Fe(II)/0.15 mM DTPA, and then ESR spectra were recorded 5 min after the start of the reaction. Lipid radical was detected in a 50-μL reaction mixture containing 100 mM POBN, 20 mM sodium dodecyl sulfate (SDS), 40 mM linoleic acid (LA), 0.11 mM Cu^{2+}, 0.1 M H_2O_2, 20 mM phosphate-buffered saline (PBS), and 20 μL of sample. The reaction was initiated by Cu^{2+}, and ESR spectra were recorded 5 min after Cu^{2+} addition. For superoxide, ESR spectra were obtained in the reaction mixture containing 0.33 mM hypoxanthine, 0.83 mM DTPA, 0.15 M DMPO, 0.083 U/mL xanthine oxidase, and a 20-μL sample. Spectra were measured 3 min after addition of xanthine oxidase (XOD) to initiate the reaction.

Mitochondrial preparation

Male Sprague-Dawley rats (180–200 grams) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). The animals were killed by decapitation after an overnight fast, and their livers were removed for immediate isolation of mitochondria. Mitochondria were isolated as described[22] with slight modification. Briefly, tissues were rinsed with saline, weighed, and put into ice-cold isolation buffer containing 0.25 mM sucrose, 10 mM Tris, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. Tissues were carefully minced and rinsed to remove residual blood, and then homogenized in 2.5 volumes of isolation buffer. The homogenate was adjusted to 8 volumes with isolation buffer and centrifuged at 1,000 $\times$ *g* for 10 min; the resultant supernatant fraction was decanted and saved, the pellet was washed once with 2 volumes of isolation buffer. The supernatant obtained after 1,000 $\times$ *g* centrifugation was combined and centrifuged at 10,000 $\times$ *g* for 10 min. The mitochondria in the pellet obtained by the 10,000 $\times$ *g* centrifiguation was resuspended with isolation buffer followed by being washed twice with the buffer. All of the above operations were carried out at 4°C. The mitochondrial protein was determined by the BCA Protein Assay kit with bovine serum albumin (BSA) as the standard. Fresh mitochondria were either used immediately for reactive oxygen species (ROS) and mitochondrial potential assays, or stored at −80°C until enzyme analysis.

ROS assay with DCF-DA in isolated rat liver mitochondria

ROS was assayed as described.[23] In brief, 0.25 mg of isolated liver mitochondria were added to 500 μL of isolation buffer with 5 mM AAPH, 5 mM pyruvate, and 10 μM DCF-DA, and then incubated with or without extract (final concentration 1, 5, 10, 20 μg/mL Regrapex-R) for 30 min at 37°C. ROS generation was recorded fluorimetrically for 30 min with an excitation wavelength of 488 nm and an emission

wavelength 525 nm by fluorometer (Flex Station II 384, Molecular Device).

Mitochondrial enzyme activity assays

For all enzyme assays, mitochondria (500 μg/mL) were dissolved in different buffers (indicated under specific enzyme assay) and exposed to a certain concentration of AAPH for 30 min or 60 min with or without the presence of Regrapex-R at 37°C. Mitochondria were then harvested for enzyme assay. NADH-CoQ oxidoreductase (complex I) activity was assayed by monitoring the reduction of DCPIP at 600 nm upon addition of assay buffer (10× buffer containing 0.5 M Tris-HCl , pH 8.1, 1% BSA, 10 μM antimycin A, 2 mM NaN_3, 0.5 mM coenzyme Q1). The final concentration of mitochondria protein was 25 μg/mL. The reaction was started by adding 200 μM NADH and scanned at 600 nm for 2 min. Assays of succinate-CoQ oxidoreductase (complex II) were performed as described.[24,25] Briefly, complex II was assayed in the assay buffer (10× buffer contain 0.5 M phosphate buffer, pH 7.8, 1% BSA, 10 μM antimycin A, 2 mM NaN_3, 0.5 mM coenzyme Q1) with mitochondria (final concentration 25 μg/mL). The reaction was started with 10 mM succinate and scanned at 600 nm for 2 min at 30°C. The pyruvate dehydrogenase (PDH) assay was carried out according to the Hinman method.[26] Briefly, the AAPH-reacted mitochondrial suspension was placed in an assay buffer containing 2.5 mM NAD^+, 0.1 mM coenzyme A, 0.2 mM TPP, 1 mM $MgCl_2$, 1 mg/ml BSA, 0.05 M Tris-HCl buffer, pH 7.8, 0.6 mM INT,and 0.1 mg/mL lipoamide dehydrogenase.The reaction was started with 5 mM pyruvate and 0.3 mM dithiothreitol, then scanned at 500 nm for 2 min. All assays were performed at 30°C.

Protein carbonyl assay in isolated rat liver mitochondria

Liver mitochondria were treated with 1 mM AAPH for 30 min at 37°C with or without extract. Protein carbonyls in mitochondria were assayed with the Oxyblot protein oxidation detection kit (Chemicon, Purchase, CA), followiong the kit guide. The carbonyl groups in the protein side chains were derivatized to 2,4,-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2, 4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by western blotting. Additional polyacrylamide resolving gels (12%, wt/vol) loaded with the same quantity of samples were electrophoresed and stained with Coomassie Brilliant Blue R250 as the loading control.

Cell viability and ROS generation in SK-N-MC cells

SK-N-MC cells were grown (Corning Glass, Corning, NY) in minimal essential medium (MEM) in a 96-well plate with 10% fetal bovine serum at 5% CO_2 and 37°C. When cells grew to 80% confluence, extract was added in serum-free medium and the cells were cultured for 24 h. Cells were then washed and exposed to 15 mM AAPH 1 h before further assays. After exposure to AAPH, cells were washed once with PBS. Cell viability was measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The ROS assay was performed as follows: Cells were incubated with 10 μM DCF-DA for 15 min, and the kinetic curve of ROS generation was recorded fluorimetrically for 30 min with an excitation wavelength of 488 nm and an emission wavelength 525 nm by fluorometer (Flex Station II 384, Molecular Device).

Drosophila culture

Transgenic α-synuclein *Drosophila* were obtained from Dr. Feany at Harvard University.[28] The Parkinson model flies express α-synuclein and produce adult-onset loss of dopaminergic neurons, filamentous intraneuronal inclusions containing α-synuclein, and locomotor dysfunction. The control non-PD flies (UAS–wild-type α-synuclein/+) and PD flies (Ddc–GAL4/+; UAS–wild-type α-synuclein/+) were maintained at 25°C on a 12-h light/dark cycle in bottles containing agar, corn meal, sucrose, water, and dried yeast medium. Propionic acid was added to prevent fungal growth. Extract was added to the medium at the final concentrations (0.08–0.64 mg/100 grams of culture) marked in the result, e.g., PD+0.08 representing flies of PD model fed with medium containing 0.08 mg extract/100 grams. UAS–wild-type α-synuclein flies were used as the basis for comparison with the PD flies because they were the female parent of PD flies and a genetically steady, constant breeding stock whose functional behavior in our standard locomotor assay described below was identical with the transgenic stock during the first 6 or 12 days after eclosion.

Drosophila climbing (negative geotaxis) assay

The climbing assay was performed as described.[29] Ten flies were placed in an empty vitreous 110- × 27-mm vial; a horizontal line was drawn 80 mm above the bottom of the vial. After the flies had acclimated for 10 min at room temperature, every group was assayed at random, to a total of 10 trials for each. The procedure involved gently tapping the flies down to the bottom of the vial. The number of flies above the mark of the vial was counted after 10 s of climbing and was repeated for 10 times to get the mean number above the mark of flies in this vial. These values were then averaged, and a group mean and standard error were obtained. The resulting mean was used as the overall value, that is to say exponent of climbing ability to be discussed for each single group of flies on a particular day. Where appropriate, the mean values of various fly groups were statistically compared using an unpaired group of the Student *t*-test. All behavioral studies were performed in an isolation room at 25°C, 60–70% humidity under standard lighting conditions.

Drosophila lifespan determination

Flasks containing the desired stocks were emptied, leaving pupa to emerge (eclose) as adults. Newly eclosed flies of both the non-PD control and PD strains were placed in culture tubes (10 flies per tube) that contained Regrapex-R–treated food, which was renewed every 3 days. Control organisms were treated similarly, except that the medium was extract free. The following day, the number of dead flies was recorded at 3-day intervals until the last one died.

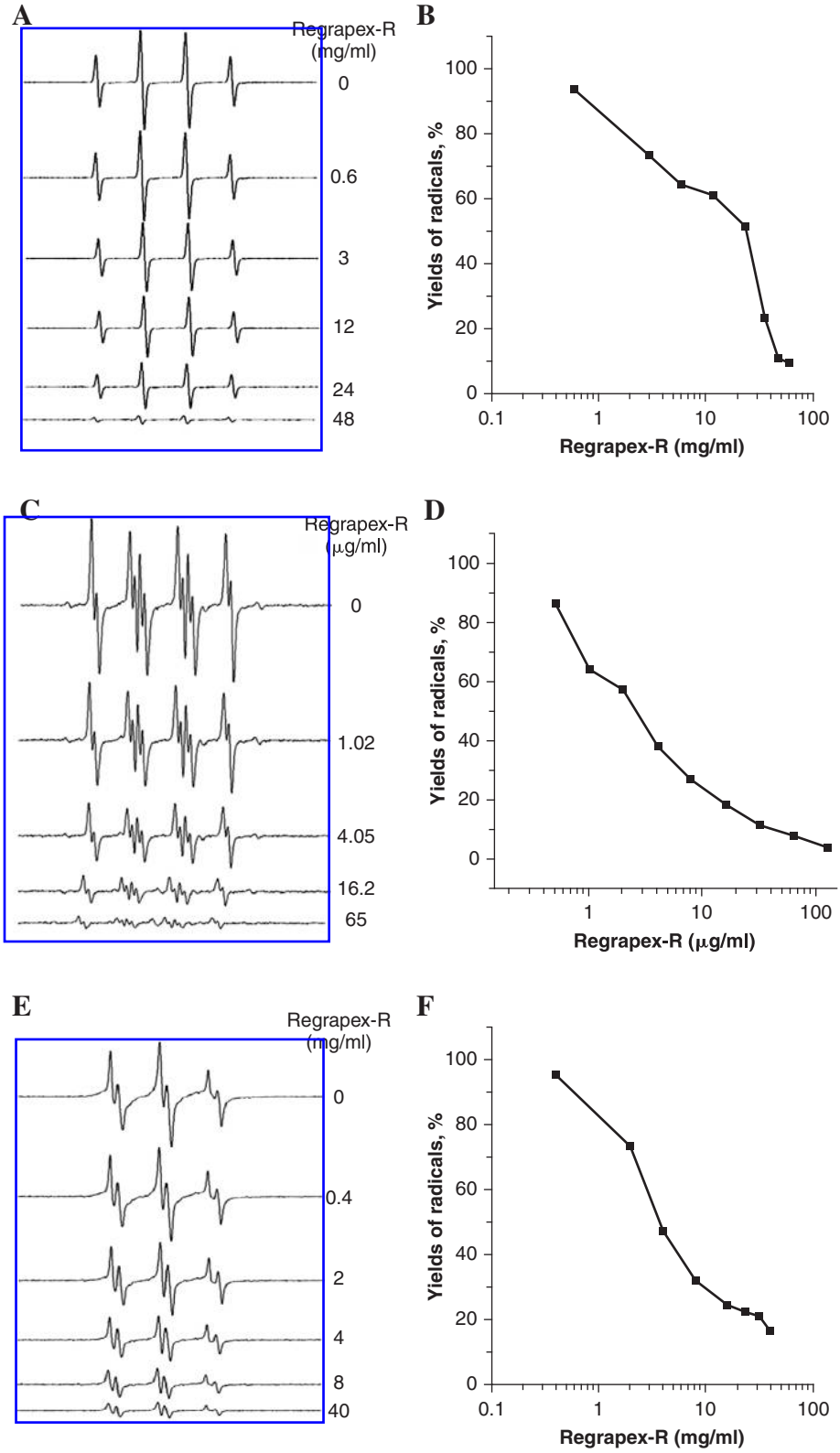

FIG. 1. Effects of Regrapex-R on hydroxyl, superoxide, and lipid radicals detected by electron spin resonance (ESR). (**A**) ESR spectra of hydroxyl radicals recorded 5 min after reaction initiation from a phosphate-buffered saline (PBS) solution (0.2 M, pH 7.4) containing 90 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 0.3 mMFe^{2+}, 100 mM hydrogen peroxide (H_2O_2). (**B**) Quantitative result of (**A**). (**C**) ESR spectra of superoxide radicals recorded 3 min after reaction initiation from a PBS solution (0.1 M, pH 7.4) containing 0.33 mM hypoxanthine, 0.83 mM DEPAPAC, 0.15 M DMPO, 0.083 units/mL xanthine oxidase, and 20 μL sample (in dimethylsulfoxide [DMSO]). (**D**) Quantitative result of (**C**). (**E**) ESR spectra of lipid radicals recorded 5 min after reaction initiation from a PBS solution (0.2 mol/L, pH 7.4) containing 90 mM 2-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN), 40 mM linoleic acid, 50 mM sodium dodecyl sulfate (SDS), 0.1 mM Cu^{2+}, 100 mM H_2O_2. (**F**) Quantitive result of (**E**). The results were means of duplicate determinations.

Table 1. Comparison of the 50% Free Radical Scavenging Concentration of Regrapex-R with Known and Specific Scavengers (mg/mL)

Radicals	*Regrapex-R*	*EPC-K1*	*Resveratrol*	*a-Tocopherol*
Hydroxyl	24.6	21.8		
Superoxide	0.0026	23.50	1.7	
Lipid	3.68	2.33		7.92

Hydroxyl, superoxide, and lipid radicals were detected using spin trapping agents with ESR spectrometer. The half-maximal inhibitory concentrations (IC_{50}) of the extract and other scavengers were calculated by linear regression of log-dose versus radical yields.

EPC-K1, Free radical scavenger and antioxidant constituted by vitamins E and C; ESR, electron spin resonance.

Statistical analyses

Data of *in vitro* assays were represented as mean ± SD of three independent experiments, and each experiment was performed in triplicate. A significant difference was calculated by using the Student *t*-test between the control and the AAPH model, and by using one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test between the AAPH model and Regrapex-R–treated groups. Data of fly locomotion assays are shown as mean ± SD. Statistical comparisons were performed by using one-way ANOVA and *post hoc* significance testing with the Dunnett multiple comparison test. The lifespan of the flies was analyzed using log rank test, and $p < 0.05$ was considered statistically significant.

Results

Regrapex-R effects on free radical scavenging

Regrapex-R showed a dose-dependent scavenging effect on hydroxyl radical (Fig. 1a,b), superoxide radical (Fig. 1c,d), and lipid radical (Fig. 1e,f). The 50% free radical scavenging concentrations (half-maximal inhibitory concentration, IC_{50}) of the extract are 24.6, 0.0026, and 3.68 mg/mL for hydroxyl, superoxide, and lipid radicals, respectively. The free radical scavenging effects were compared with some well-known and specific scavengers, as shown in Table 1. The IC_{50} of Regrapex-R for hydroxyl and lipid radicals are at a similar level to those of EPC-K1, a well-known

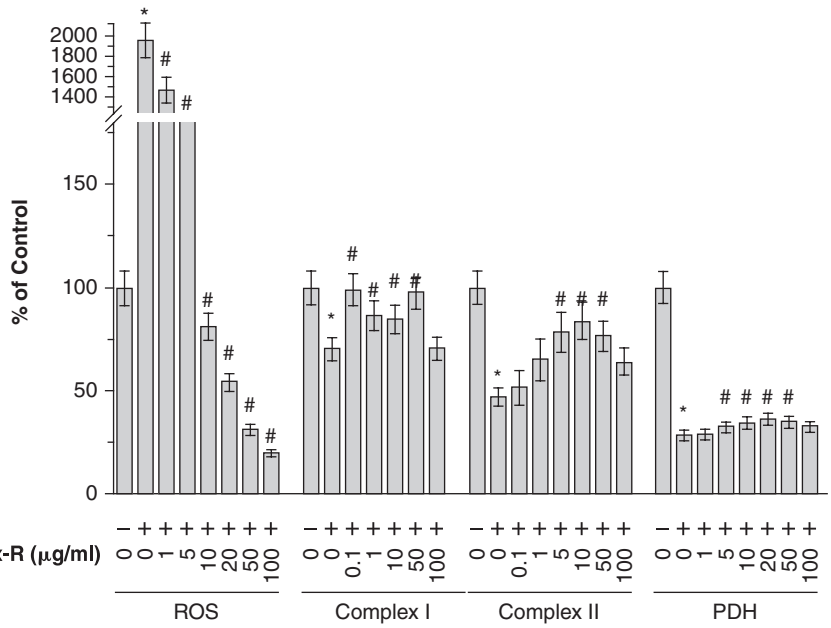

FIG. 2. Effects of Regrapex-R on 2,2′-azobis (2-amidino propane) dihydrocholoride (AAPH)-induced increase of reactive oxygen species (ROS) and mitochondrial enzymes dysfunction in liver mitochondria. For the ROS assay, 0.25 mg of isolated liver mitochondria were added to 500 μL of isolation buffer containing 5 mM AAPH, 2 mM pyruvate, and 10 μ DCF-DA, and then incubated with or without Regrapex-R (final concentration 1, 5, 10, or 20 μg/mL) 30 min at 37°C. The kinetic curve of ROS generation was recorded fluorimetrically for 30 min with an excitation 488 nm and an emission 525 nm by fluorometer (Flex Station II 384, Molecular Device). For the enzymes assay, isolated rat liver mitochondria (0.5 mg/mL) were incubated with various concentrations of Regrapex-R followed by 5 mM AAPH (for PDH), 10 mM AAPH (for complex I), or 15 mM AAPH (for complex II) exposure at 37°C for 60 min (for complex I) or 30 min (for complex II, PDH). The complex activity was measured by scanning the samples in the reaction buffers with a microplate reader at 600 nm for 2 min, and PDH activity was measured at 500 nm for 2 min as described in Materials and Methods. All values are mean ± SD of three independent experiments of three replicates each. Asterisk shows significant differences between control and AAPH model using the Student *t*-test (*) $p < 0.05$). (#) Significant difference between AAPH and Regrapex-R–treated AAPH mitochondria using one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test; (#) $p < 0.05$.

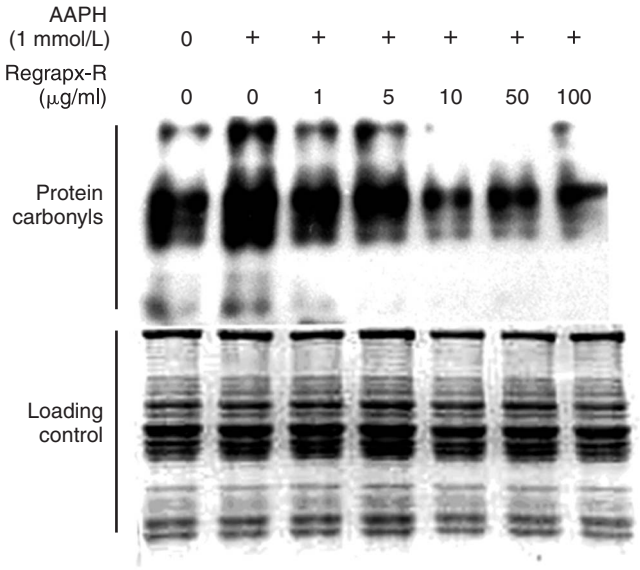

FIG. 3. Effects of Regrapex-R on 2,2′-azobis (2-amidino propane) dihydrocholoride (AAPH)-induced increase of protein carbonyls in isolated liver mitochondria. Isolated rat liver mitochondria (4 mg/mL) were incubated with various concentrations of Regrapex-R and 1 mM AAPH at 37°C for 30 min. Protein carbonyls were detected by western blotting using the Oxyblot protein oxidation detection kit. Another polyacrylamide resolving gel (12%, wt/vol) loading the same quantity of samples was electrophoresed and stained with Coomassie Brilliant Blue R250 as the loading reference.

free radical scavenger and antioxidant constituted by vitamins E and C,[30,31] while 9,038-fold more potent than EPC-K1 for superoxide radical. Regrapex-R was about 653-fold more potent for superoxide radical scavenging, compared with resveratrol,[32] a caloric restriction mimetic agent able to extend the lifespan of *Drosophila*.[33] Regrapex-R was 2-fold more potent for lipid radical scavenging when compared with α-tocopherol.

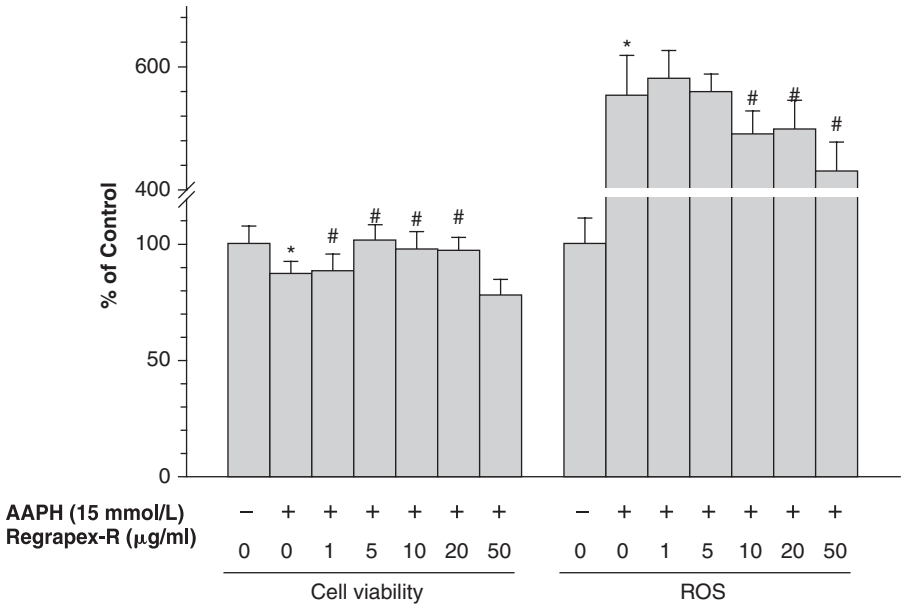

FIG. 4. Effects of Regrapex-R on the cell viability and reactive oxygen species (ROS) generation in SK-N-MC cells damaged by 2,2′-azobis (2-amidino propane) dihydrocholoride (AAPH). Cells (5,000 cells per well) in 96-well plate were grown for 72 h until 80% confluent. Regrapex-R was added in serum-free media and cultured for 24 h. Cells were washed, exposed to 15 mM AAPH for 1 h, washed again, then subjected to 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or ROS assay. The data shown were mean ± standard deviation (SD) of three independent experiments of six duplicates. The asterisk indicates a significant difference between the control and AAPH model using the Student *t*-test; (*) $p < 0.05$). (#) Significant difference between AAPH and Regrapex-R–treated AAPH-cells using one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test; (#) $p < 0.05$).

Effect of Regrapex-R on AAPH-induced ROS generation in isolated liver mitochondria

We tested the protective effects of Regrapex-R on AAPH, a lipid-like oxidant generator that induced mitochondrial damage in isolated rat liver mitochondria. As shown in Fig. 2, AAPH greatly increased ROS generation, and Regrapex-R demonstrated a dose-dependent inhibition on AAPH-induced ROS increase in the liver mitochondria at the dose range of 1–100 μg/mL.

Effect of Regrapex-R on AAPH-induced inactivation of mitochondrial enzymes in isolated liver mitochondria

As shown in Fig. 2, mitochondrial complex I lost about 30% activity following exposure to 10 mM AAPH for 1 h. Regrapex-R showed a protective effect at the concentrations of 0.1–50 μg/mL. Similarly, mitochondrial complex II lost 50% activity in the insult of 15 mM AAPH for 30 min, and pretreatment of Regrapex-R at the range of 5–50 μg/mL showed significant protection against AAPH-induced damage (Fig. 2). As for PDH, a key dehydrogenase located in mitochondria, Regrapex-R, also showed a dose-dependent protection at the dose range of 5–50 μg/mL (Fig. 2).

Effect of Regrapex-R on AAPH-induced protein oxidation in isolated liver mitochondria

As shown in Fig. 3, AAPH insult resulted in a significant increase of protein carbonyl in liver mitochondria, which can be remarkably attenuated by Regrapex-R in a dose-dependent manner at 1 μg/mL to 100 μg/mL (Fig. 3).

Effect of Regrapex-R on cell viability and ROS in SK-N-MC cells

SK-N-MC cell viability showed a 15% decrease when cells were challenged with AAPH (15 mM) for 1 h (Fig. 4), and

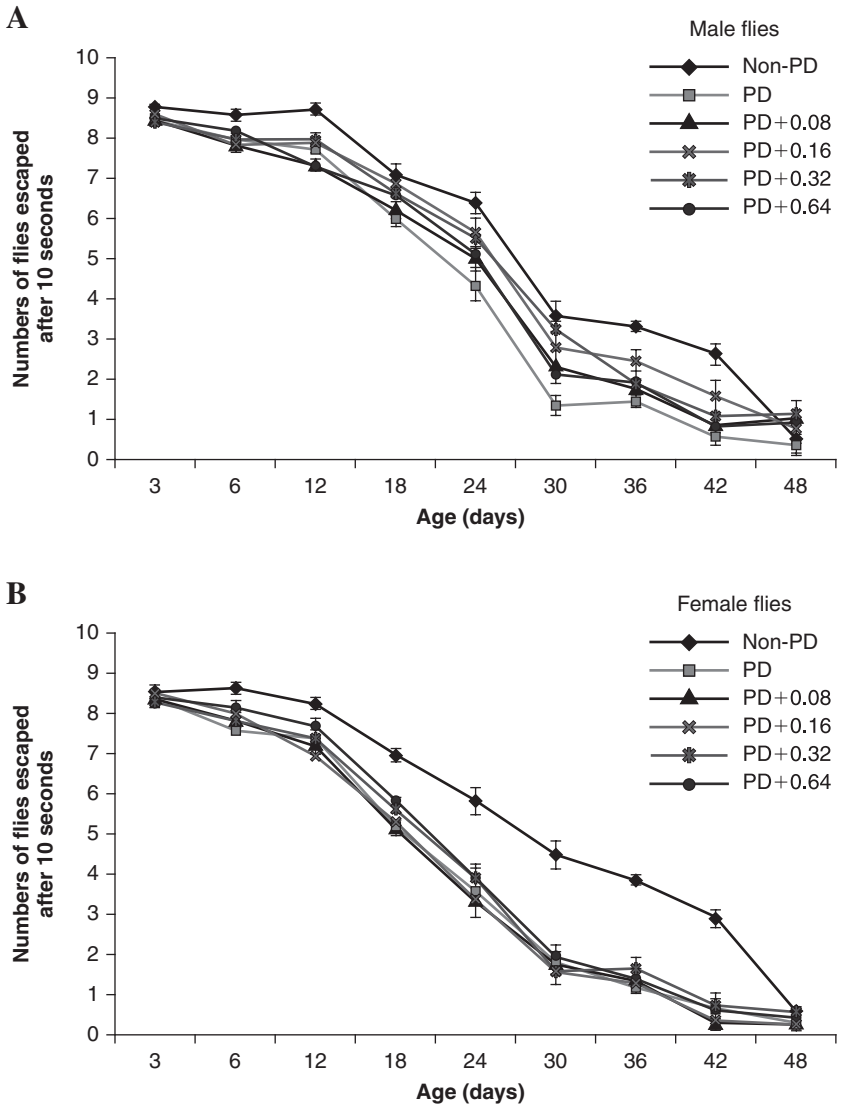

FIG. 5. Effects of Regrapex-R on the climbing response in *Drosophila*. (**A**) Male *Drosophila*; (**B**) Female *Drosophila*. The negative geotaxis climbing assay was performed as described in *Materials and Methods*. The data shown were means ± standard deviation (SD) with the 230 flies each group in the beginning of the experiment. PD, Parkinson disease. (color images available online at www. liebertonline.com/rej)

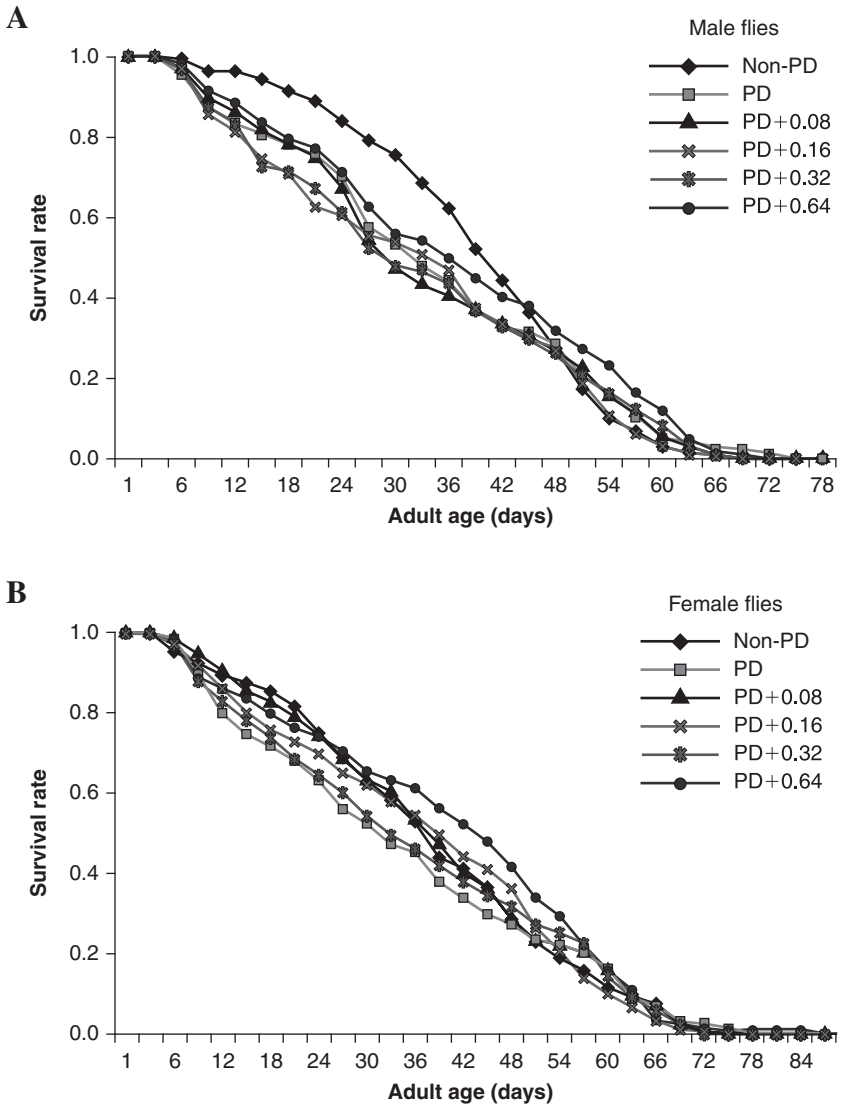

FIG. 6. Effects of Regrapex-R on the survival rate in Drosophila. (**A**) Male *Drosophila*. (**B**) Female *Drosophila*. Newly eclosed flies of both the non-Parkinson disease (PD) control and PD strains were placed in culture tubes (10 flies per tube) that contained food with or without Regrapex-R. Food was renewed every 3 days. The number of dead flies was recorded at 3-day intervals until the last fly died. (color images available online at www. liebertonline.com/rej)

pretreatment with 5–20 μg/mL Regrapex-R for 24 h completely protected cells against oxidative insult. However, the protective effect disappeared at the higher Regrapex-R concentration of 50 μg/mL (Fig. 4). AAPH-induced (15 mM for 1 h) ROS generation was greatly elevated to 5.5-fold higher than that of control cells without insult, and Regrapex-R significantly inhibited the AAPH-induced ROS generation at the concentration $\geq$10 μg/mL (Fig. 4).

Regrapex-R effects on the climbing activity and life span of Drosophila PD model

As shown in Fig. 5, the climbing response of the non-PD and PD flies (Fig. 5a, male; Fig. 5b, female) decreased progressively with age. However, the climbing response of the male PD flies was significantly lower than those of the non-PD flies at the age range of 3 days to 42 days (vs. non-PD, $p < 0.05$), and female PD flies showed a similar decline of locomotion at the age range of 6 days to 42 days compared with non-PD control (vs. non-PD, $p < 0.05$). Regrapex-R was mixed in the culture of the test groups of transgenic flies at the concentrations of 0.08–0.64 mg/100 grams of culture medium (corn medium). These levels were selected on the basis of previous studies indicating that they were effective but not toxic and nonlethal. Noticeably, at the concentration range of 0.16–0.64 mg/100 grams of culture medium as shown in Fig. 5a, Regrapex-R significantly improved the climbing response in male PD flies at the age range from 18 days up to 42 days (vs. PD without treatment, $p < 0.05$). However, no consecutive significant differences between PD and Regrapex-R–treated PD female flies were observed.

As for lifespan evaluation in male flies (Fig. 6a), transgenic PD flies showed a shorter lifespan than that of con-

trol α-synuclein flies (vs. non-PD, $p < 0.01$). Among four doses of Regrapex-R treatments, one dose at 0.64 mg/100 grams of culture significantly extended lifespan of PD male flies (vs. PD without treatment, $p < 0.05$). In contrast to the effect on male PD flies, Regrapex-R did no improve the climbing response in female flies; however, as shown in Fig. 6b, Regrapex-R exhibited an effect on extending the average lifespan in female PD flies at the doses from 0.16 mg to 0.64 mg/100 grams of medium (vs. PD without treatment, $p < 0.05$).

Discussion

Our data showed that Regrapex-R, a combination botanical formulation comprised of an extract of whole grape (*Vitis vinifera*) and *Polygonum cuspidatum* possesses potent antioxidant activity, protects mitochondria from oxidative damage, and is an effective agent in ameliorating the loss of locomotor function in a *Drosophila* model for PD. We believe this is the first study to demonstrate a direct neuroprotective effect of a grape-based extract in a genetic model of PD.

PD, the second most common neurodegenerative disorder after Alzheimer disease, is characterized by several abnormalities, including inflammation, mitochondrial dysfunction, iron accumulation, and oxidative stress. Many pharmaceutical intervention strategies are targeted to one or more of these abnormalities by focusing on the molecular pathways underlying the dysfunction and the role that these pathways may play in disease initiation and progression. Most therapeutic strategies currently rely on providing protection against the massive degenerative loss of dopamine neurons, particularly in the substantia nigra. Whereas classic therapy relies on administration of levodopa, the efficacy of this agent declines as PD progresses. Newer strategies aimed at rescue of nigral dopamine neurons from progressive cell death are currently being explored. One pathway target of primary interest and explored in this study is oxidative stress, a dysfunction that can be reduced by agents with potent free radical scavenging capabilities.

The results of our free radical scavenging experiments agree with previous reports of the potent antioxidant activity associated with polyphenols found in grape extracts.[34] However, we observed particularly potent activity against the superoxide radical where Regrapex-R was 653-fold more potent than resveratrol and >9,000 fold more potent than EPC-K1, a combination of α-tocopherol and ascorbic acid.

It is well documented that mitochondria are the major source of intracellular ROS concomitant with the oxygen phosphoralytion. The major single-organ oxygen consumers are the liver and brain, consuming 20.4% and 18.4%, respectively. In our previous *in vitro* study, we found that the endogenous ROS (RFU/mg of protein fluorescently detected with DCF-DA) in freshly isolated murine liver mitochondria exhibit a 30 times higher level than that in brain mitochondria and also showed significant vulnerability to external oxidatative insults, such as AAPH, H_2O_2, etc. (unpublished). Therefore, the liver mitochondria can be served as an optimal subcellular system to evaluate the antioxidative capacity of the potential antioxidants. In the current study, following the evaluation of the specific activity to scanvenging hydroxyl radicals, lipid radicals and superoxides by ESR assays, the impact of antioxidative activity on the biological effect was investigated in isolated rat liver mitochondria with AAPH insult. We indeed observed a protective effect of the extract against oxidative damage by AAPH on maintaining activities of mitochondrial enzymes (complexes I and II and PDH), suppressing excessive ROS and resisting protein oxidative modification. The antioxidative benefits of the extract were further verified on human SK-N-MC neuroblastoma cells, which retain cell viability and have a lower level of ROS than vehicle-treated cells following exposure to the toxin.

Oxidative stress has been hypothesized to be linked to both the initiation and the progression of PD.[13] Genetic mutation of α-synuclein, a major component of Lewy bodies in sporadic PD, may increase neuronal vulnerability to cellular oxidative stress in PD pathogenesis.[32] In that potent antioxidant capacity of the extract as we investigated the *in vitro* system, we evaluated the potential effects of grape extract on the locomotion and lifespan of genetic *Drosophila* model of PD developed by Feany and Bender in 2000.[28] In this model, normal and mutant forms of α-synuclein were expressed in *Drosophila*, producing adult-onset loss of dopaminergic neurons and filamentous intraneuronal inclusions containing α-synuclein with consequent locomotor dysfunction. A recent proteomic analysis of this panneural expression of human wild type (WT) α-synuclein in the transgenic flies showed a differential expression of proteins indicating a perturbation of molecular pathways involving metabolism and signaling.[35] Gene expression changes for genes in these molecular pathways have been shown to be greatest in this model at the presymptomatic stage, when the potential for neuroprotection is greatest, thus validating this model for identifying potential targets for neuroprotective strategies.[36] A relatively straightforward negative geotaxis assay can be performed to determine whether or not a variety of compounds or drugs incorporated into fly food prevent the progressive loss of climbing ability of the α-synuclein transgenic flies.

Our results showed a significant improvement in the climbing response of PD flies at a Regrapex-R dose range of 0.16–0.64 mg/100 grams of culture medium. This effect was also demonstrated to show a gender bias because only male flies fed grape extract showed an improvement in climbing, whereas female flies did not. These results, taken together, suggest that grape extract fortified with resveratrol may exert its neuroprotective effect through prevention of oxidative damage via potent ROS scavenging ability.

Grape extract extended the average lifespan of female PD flies at the concentration 0.64 mg/100 grams of culture medium. This effect, like that observed in the climbing assay, also showed a gender bias with no extension of lifespan observed in the male PD flies (except of 0.64 mg/100 grams treatment). Specific metabolic signaling pathways and differentiated gene expression in different genders may be involved in those sex-specific protective effects in flies.[37–40] Further investigation will be directed at revealing this interesting phenomenon.

There is a great deal of interest in identifying agents that have an effect on aging. Notably, resveratrol, an enriched component of the Regrapex-R formulation and a common component of grapes, has been demonstrated to extend

lifespan in a variety of organisms, including yeast, nematodes, and *Drosophila*.[33] Our results are consistent with these findings, but suggest that grape polyphenols should be the focus of further research in this area because a direct effect of grape polyphenols alone on lifespan extension has not yet been demonstrated.

Results from these studies contribute to our understanding of the role of polyphenols in protecting against neurodegenerative disease. The data suggest that polyphenols exert their protective effects in a manner consistent with the most recent proposed mechanisms underlying the pathogenesis of PD. Regrapex-R and similar polyphenol-rich combination botanical products should be further evaluated as therapeutic strategies aimed at prevention and treatment of neurodegenerative diseases.

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References

1. da Costa ESO, Knoll R, Jager M. Personalized nutrition: An integrative process to success. Genes Nutr 2007;2:23–25.
2. Cui J, Juhasz B, Tosaki A et al. Cardioprotection with grapes. J Cardiovasc Pharmacol 2002;40:762–769.
3. Du Y, Guo H, Lou H. Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. J Agric Food Chem 2007;55:1695–1701.
4. Chaves AA, Joshi MS, Coyle CM et al. Vasoprotective endothelial effects of a standardized grape product in humans. Vascul Pharmacol 2009;50:20–26.
5. Vinson JA, Mandarano MA, Shuta DL et al. Beneficial effects of a novel IH636 grape seed proanthocyanidin extract and a niacin-bound chromium in a hamster atherosclerosis model. Mol Cell Biochem 2002;240:99–103.
6. Zern TL, West KL, Fernandez ML. Grape polyphenols decrease plasma triglycerides and cholesterol accumulation in the aorta of ovariectomized guinea pigs. J Nutr 2003;133: 2268–2272.
7. Soares De Moura R, Costa Viana FS, Souza MA et al. Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract. J Pharm Pharmacol 2002;54:1515–1520.
8. Badavi M, Mehrgerdi FZ, Sarkaki A et al. Effect of grape seed extract on lead induced hypertension and heart rate in rat. Pak J Biol Sci 2008;11:882–887.
9. Kaur M, Mandair R, Agarwal R et al. Grape seed extract induces cell cycle arrest and apoptosis in human colon carcinoma cells. Nutr Cancer 2008;6(Suppl 1):2–11.
10. Agarwal C, Singh RP, Agarwal R. Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release. Carcinogenesis 2002;23:1869–1876.
11. Chatelain K, Phippen S, McCabe J et al. Cranberry and grape seed extracts inhibit the proliferative phenotype of oral squamous cell carcinomas. Evid Based Complement Alternat Med 2008; Epub.
12. Wang J, Ho Epub. Zhao W et al. Grape-derived polyphenolics prevent Abeta oligomerization and attenuate cognitive deterioration in a mouse model of Alzheimer's disease. J Neurosci 2008;28:6388–6392.
13. Thomas P, Wang YJ, Zhong JH et al. Grape seed polyphenols and curcumin reduce genomic instability events in a transgenic mouse model for Alzheimer's disease. Mutat Res 2009; 661: 25–34.
14. Deshane J, Chaves L, Sarikonda KV et al. Proteomics analysis of rat brain protein modulations by grape seed extract. J Agric Food Chem 2004;52:7872–7883.
15. Yang Y, Chien M. Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Agric Food Chem 2000; 48:3990–3996.
16. Weber HA, Hodges AE, Guthrie JR et al. Comparison of proanthocyanidins in commercial antioxidants: grape seed and pine bark extracts. J Agric Food Chem 2007;55:148–156.
17. Bagchi D, Bagchi M, Stohs SJ et al. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 2000;148:187–197.
18. Sovak M. Grape extract, Resveratrol, and its analogs: A review. J Med Food 2001;4:93–105.
19. Liu J, Mori A. Antioxidant and free radical scavenging activities of Gastrodia elata Bl. and Uncaria rhynchophylla (Miq.) Jacks. Neuropharmacology 1992;31:1287–1298.
20. Liu J, Mori A. Antioxidant and pro-oxidant activities of p-hydroxybenzyl alcohol and vanillin: effects on free radicals, brain peroxidation and degradation of benzoate, deoxyribose, amino acids and DNA. Neuropharmacology 1993;32: 659–669.
21. Liu J, Mori A. Monoamine metabolism provides an antioxidant defense in the brain against oxidant- and free radical-induced damage. Arch Biochem Biophys 1993;302:118–127.
22. Long J, Wang X, Gao H et al. Malonaldehyde acts as a mitochondrial toxin: Inhibitory effects on respiratory function and enzyme activities in isolated rat liver mitochondria. Life Sci 2006;99:1466–1472.
23. Long J, Liu C, Sun L et al. Neuronal mitochondrial toxicity of malondialdehyde: inhibitory effects on respiratory function and enzyme activities in rat brain mitochondria. Neurochem Res 2009;34:786–794.
24. Long J, Ma J, Luo C et al. Comparison of two methods for assaying complex I activity in mitochondria isolated from rat liver, brain and heart. Life Sci 2009; 85: 276–280.
25. Humphries KM, Yoo Y, Szweda LI. Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. Biochemistry 1998;37:552–557.
26. Picklo MJ, Amarnath V, McIntyre JO et al. 4-Hydroxy-2(E)-nonenal inhibits CNS mitochondrial respiration at multiple sites. J Neurochem 1999;72:1617–1624.
27. Hinman LM, Blass JP. An NADH-linked spectrophotometric assay for pyruvate dehydrogenase complex in crude tissue homogenates. J Biol Chem 1981;256:6583–6586.
28. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. Nature 2000;404:394–398.
29. Pendleton RG, Parvez F, Sayed M et al. Effects of pharmacological agents upon a transgenic model of Parkinson's disease in Drosophila melanogaster. J Pharmacol Exp Ther 2002;300:91–96.
30. Noda Y, Anzai K, Mori A et al. Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. Biochem Mol Biol Int 1997;42:35–44.
31. Wei T, Chen C, Li F et al. Antioxidant properties of EPC-K1: A study on mechanisms. Biophys Chem 1999;77:153–160.
32. Leonard SS, Xia C, Jiang BH et al. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular

responses. Biochem Biophys Res Commun 2003;309:1017–1026.
33. Wood JG, Rogina B, Lavu S et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 2004;430:686–689.
34. Yamaguchi F, Yoshimura Y, Nakazawa H et al. Free radical scavenging activity of grape seed extract and antioxidants by electron spin resonance spectrometry in an H(2)O(2)/NaOH/DMSO system. J Agric Food Chem 1999;47:2544–2548.
35. Xun Z, Kaufman TC, Clemmer DE. Proteome response to the panneural expression of human wild-type alpha-synuclein: A Drosophila model of Parkinson's disease. J Proteome Res 2008;7:3911–3921.
36. Scherzer CR, Jensen RV, Gullans SR et al. Gene expression changes presage neurodegeneration in a Drosophila model of Parkinson's disease. Hum Mol Genet 2003;12:2457–2466.
37. Bauer M, Katzenberger JD, Hamm AC et al. Purine and folate metabolism as a potential target of sex-specific nutrient allocation in Drosophila and its implication for life-span-reproduction tradeoff. Physiol Genomics 2006;25:393–404.
38. Yamamoto D. Brain sex differences and function of the fruitless gene in Drosophila. J Neurogenet 2008; 22:1–24.
39. Sorensen JG, Kristensen TN, Kristensen KV et al. Sex specific effects of heat induced hormesis in Hsf-deficient Drosophila melanogaster. Exp Gerontol 2007;42:1123–1129.
40. Tricoire H, Battisti V, Trannoy S et al. The steroid hormone receptor EcR finely modulates Drosophila lifespan during adulthood in a sex-specific manner. Mech Ageing Dev 2009;130: 547–552.

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