A botanical containing freeze dried açai pulp promotes healthy aging and reduces oxidative damage in *sod1* knockdown flies

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Abstract Superoxide dismutase 1 (SOD1), a critical enzyme against oxidative stress, is implicated in aging and degenerative diseases. We previously showed that a nutraceutical containing freeze-dried açai pulp promotes survival of flies fed a high-fat diet or *sod1* knockdown flies fed a standard diet. Here, we investigated the effect of açai supplementation initiated at the early or late young adulthood on lifespan, physiological function, and oxidative damage in *sod1* knockdown flies. We found that Açai supplementation extended lifespan even when started at the age of 10 days, which is the time shortly before the mortality rate of flies accelerated. Life-long açai supplementation increased lifetime reproductive output in *sod1* knockdown flies. Our molecular

studies indicate that açai supplementation reduced the protein levels of genes involved in oxidative stress response, cellular growth, and nutrient metabolism. Açai supplementation also affected the protein levels of ribosomal proteins. In addition, açai supplementation decreased the transcript levels of genes involved in oxidative stress response and gluconeogenesis, while increasing the transcript levels of mitochondrial biogenesis genes. Moreover, açai supplementation reduced the level of 4-hydroxynonenal-protein adducts, a lipid peroxidation marker. Our findings suggest that açai supplementation promotes healthy aging in *sod1*-deficient flies partly through reducing oxidative damage, and modulating nutrient metabolism and oxidative stress response pathways. Our findings provide a foundation to further evaluate the viability of using açai as an effective dietary intervention to promote healthy aging and alleviate symptoms of diseases with a high level of oxidative stress.

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Introduction

Aging is associated with a decline in physiological function and an increased susceptibility to disease (Fontana et al. 2010). The free radical theory of aging posits that cumulative oxidative damage to cellular

macromolecules contributes greatly to this overall degeneration of the organism (Harman 1956; Perez et al. 2009). Many genetic and environmental factors, particularly factors which modulate oxidative stress response pathways, have an impact on the process of aging and aging-associated diseases, such as neurodegenerative diseases (Fontana et al. 2010). Conserved pathways that are involved in modulating lifespan have been identified ranging from yeast, worms, flies, to rodents. Among these pathways are the target of rapamycin (TOR) pathway involved in regulating cell growth and responding to nutrients and redox status and the Jun kinase (JNK) signaling pathway involved in modulating cellular oxidative stress response (Fontana et al. 2010; Karpac and Jasper 2009). Genes in these pathways are implicated in human longevity (Barbieri et al. 2010). Degenerative diseases are often associated with higher levels of oxidative damage in patients. Interventions to alleviate oxidative damage have been shown to delay the onset of age-associated degenerative diseases and improve health in numerous animal models of human disease (Perez et al. 2009; Salmon et al. 2010). For example, overexpression of glutathione S-transferase S1, a member of important detoxification enzymes, reduces dopaminergic neuron degeneration in a *Drosophila* model of Parkinson's disease (Whitworth et al. 2005).

Nutraceuticals made from various botanicals have been shown to provide numerous health benefits, including prevention of microbial infection, reduction of inflammation, prevention of cancer and cardiovascular diseases, and promotion of healthy aging (Joseph et al. 2009; Liu 2004). Many nutraceuticals contain high content of phytochemicals, such as polyphenols, which possess high antioxidant activity and other functions to regulate activities of various enzymes and genes involved in modulating lifespan and age-associated diseases (Pan et al. 2009; Joseph et al. 2009; Liu 2004). A number of such nutraceuticals have been shown to extend lifespan and healthspan in diverse model organisms. To name a few, curcumin, a natural polyphenol compound found in the spice turmeric, extends lifespan in flies and worms by reducing oxidative damage and maintaining homeostasis of protein turnover (Lee et al. 2010; Alavez et al. 2011). Nectarine, a globally grown and consumed fruit, is high in polyphenols and promotes healthy aging in flies on a regular or high-fat diet by reducing oxidative stress (Boyd et al. 2011). Catechins from green tea

when supplemented to mice in their late life can decrease oxidative damage and delay brain senescence (Unno et al. 2008). Phytochemicals from apples have neuroprotective effects by suppressing amyloid-protein aggregation in mammalian neuronal PC-12 cells and extend lifespan in worms and flies partly by modulating oxidative stress response genes (Toda et al. 2011; Peng et al. 2011). These studies suggest that nutraceuticals may serve as viable and cost-effective interventions to promote healthy aging in humans.

One challenge to the treatment of age-associated diseases, such as degenerative diseases, is that the symptoms of these diseases manifest often after the disease is in an advanced stage (Barak and Aizenberg 2010). Age-associated degenerative diseases are commonly associated with high oxidative damage in the cell. Very few therapeutic approaches are available to effectively treat degenerative diseases without many side effects (Hung et al. 2010; Ang et al. 2010). Treatment with simple antioxidants does not necessarily provide any health benefits and is even detrimental in some cases to patients with degenerative diseases (Vina et al. 2011). Here, we initiated a study to test the hypothesis that antioxidant- and phytochemical-rich nutraceuticals can be utilized in the late-life intervention to reduce oxidative damage and promote healthy aging in patients with a high level of oxidative stress. To this end, we employed a *Drosophila* model with superoxide dismutase 1 (*sod1*) deficiency. SOD1 is a key enzyme for scavenging superoxide radicals in the cell and is implicated in amyotrophic lateral sclerosis (ALS), a motor neuron degenerative disease (Fridovich 1995; Rosen et al. 1993). *sod1* mutant flies have increased oxidative damage and live shorter compared to wild-type flies (Phillips et al. 1989, 1995). Açai (*Euterpe oleracea*) is a fruit abundant in the Amazon River region, which contains high levels of phytochemicals (Schauss et al. 2006a, b). Emerging lines of evidence show that freeze-dried açai pulp possesses high antioxidant and anti-inflammation bioactivities, and anti-cancer and anti-cardiovascular disease properties in cell and animal models of human diseases (Poulose et al. 2012; Feio et al. 2011; Xie et al. 2011a; Xie et al. 2012; Stoner et al. 2010). We previously demonstrated that life-long supplementation of freeze dried açai pulp extends lifespan in wild-type flies on a high-fat diet and *sod1* knockdown flies on a standard diet (Sun et al. 2010). In this study, we tested whether açai supplementation during the

later life is sufficient to promote survival in *sod1* knockdown flies. We further investigated the effect of açai supplementation on reproductive output and locomotor activity in *sod1* knockdown flies. Moreover, we investigated the molecular mechanisms underlying the health promoting-properties of the açai pulp extract. Our findings reveal the effectiveness of açai supplementation in promoting healthy aging in oxidative stressed animals.

Materials and methods

Fly stocks and culture

Both da-Gal4 (w^{1118} ; P{w[+mW.hs]=GAL4-da.G32},3) and UAS-*sod1IR* (w^{1118} ; P{UAS-Sod1.IR} F103/SM5) lines were backcrossed with w^{1118} for more than five generations to minimize variations in genetic background. These stocks were maintained as heterozygous lines, da-Gal4/+ and UAS-*sod1IR*/+, respectively, before being used in all the experiments. UAS-*sod1IR* carries a transgene with inverted repeat sequences of *sod1* under the control of the UAS promoter. *sod1* was knocked-down using the Gal4-UAS system in combination with the RNA interference technique as described previously (Sun et al. 2010). Specifically, *sod1* knockdown flies, called *sod1RNAi* (UAS-*sod1IR*/+; da-Gal4/+), were generated from mating da-Gal4/+ flies with UAS-*sod1IR* homozygous flies from the UAS-*sod1IR*/+ heterozygous line. Both da-Gal4/+ and UAS-*sod1IR*/+ lines shared similar genetic background with *sod1RNAi* flies and were used as wild-type control flies. All fly stocks were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN) and maintained on cornmeal medium at room temperature. All fly cultures for various assays were conducted at $25 \pm 1^\circ\text{C}$, $60 \pm 5\%$ humidity and a 12:12-h light/dark cycle.

The standard cornmeal food was made from cornmeal, sugar, yeast and agar according to the published protocol (Ashburner 1989). The standard sugar-yeast extract (SY) diet contained 10 % sugar and 10 % yeast extract, and 1.5 % agar (Bass et al. 2007). Freeze-dried açai pulp has been described previously (Sun et al. 2010), and was kindly provided by Dr. Alexander G. Schauss at AIBMR Life Sciences, Inc. (Puyallup, WA). The açai pulp was stored at -80°C and ground into finer particles with a pestle and mortar to increase

solubility before being added to the SY diet to final concentrations of 2 and 4 % (w/v).

Reproduction and lifespan and assays

Parental flies were grown on cornmeal medium and progeny of mixed sex were collected within 24 h after eclosion into the bottles with the SY diet. Flies were allowed to mate for 24 h in the SY bottles, and then sorted by sex under light CO_2 anesthesia into vials with the SY diet. Only females were used in this study since our previous results indicated that females were more responsive to açai supplementation than males in terms of lifespan extension (Sun et al. 2010). For life-long intervention, sorted flies were maintained in vials with the SY diet each containing five females for another 24 h, and then separated into three treatment groups, one on the standard SY diet and two other groups on the SY diet supplemented with 2 and 4 % açai. Fresh food was provided to flies once every 2–3 days. Dead flies and the number of eggs laid were counted at each transfer to determine lifetime egg production. Egg production during each transfer interval was calculated by dividing the total number of egg production by the number of survived flies at each transfer. The lifetime egg production was calculated by dividing the lifetime egg production in each vial by the number of females at the initiation of the experiment, which are five flies per vial. The reproduction assay was repeated six times in separate vials.

For later life intervention, flies were maintained in vials with the SY diet without açai until they reached the age of 10 days. The 10-day-old flies were then separated into two treatment groups, one on the standard SY diet and the other on the standard diet supplemented with 2 % açai. Fresh food was provided to flies once every 2–3 days and dead flies were counted at each transfer to measure lifespan. Each lifespan assay was conducted with flies in seven to eight vials each with approximately 20 flies.

Food intake and locomotion assays

The capillary feeder method (CAFE) was used to measure food intake with minor modification as described previously (Sun et al. 2010; Ja et al. 2007). Thirty-two female flies were treated with the standard SY diet supplemented or not with 2 % açai for 11 days. The food used in the CAFE method contained 10 %

sugar and 10 % yeast extract, supplemented with or without 2 % freeze-dried açai pulp (w/v). The food contained no agar so that the food would remain liquid. Each feeding chamber contained two flies and eight chambers were set up for each dietary treatment. Two chambers containing no flies were set up to account for evaporation of the liquid food. Daily food intake was calculated by averaging the food intake from eight replicates for each dietary treatment.

The *Drosophila* Activity Monitoring System (DAMS) from Trikinetics (Waltham, MA, USA) was used to monitor spontaneous locomotor activity of female *sod1* knockdown flies treated for 11 days with the standard SY diet supplemented with or without 2 % açai. Six tubes each with approximately ten flies were set up for each treatment group. The glass tube containing flies and the solid food on the bottom was inserted into the DAMS, which has multiple infrared beams across the center of the vial. The number of beam breaks was recorded every 20 s over a 24-h period. After 24 h, the assay was stopped and the number of dead flies was counted in each vial. Locomotor activity was calculated by averaging the total number of beam breaks per fly during the 24-h period from six replicates per dietary treatment.

Proteomic analysis

Approximately 20 *sod1*/RNAi female flies of 14-day-old fed the SY diet supplemented with or without 2 % açai were collected and immediately frozen in liquid nitrogen. Fly heads were separated from bodies using a sieve. To isolate protein, fly bodies were homogenized in ice-cold PBS buffer with protease inhibitor cocktail using a glass dounce homogenizer. Each homogenate was centrifuged at $2,500 \times g$ at 4°C for 10 min. The supernatant was collected as the protein sample and the pellet was discarded. Protein concentration was determined with the BCA protein assay kit according to the manufacturer's suggested protocol (Thermo Fisher, cat. no. 23225). Protein samples were analyzed by a LC/MS/MS proteomic method as described previously (Wu et al. 2007). Specifically, the samples were reduced with 10 mM DTT at 60°C for 1-h, alkylated with 55 mM iodoacetamide at room temperature in the dark for 0.5 h, and then trypsinized at 37°C for 16–18 h using a protein/trypsin ratio of ~50. The digests were analyzed using a Thermo Fisher Scientific linear ion trap (LTQ XL) by C18 reversed phase chromatography. Peptides were then loaded onto a trap cartridge (Agilent, Palo Alto,

CA) at a flow rate of 2 μ L/min. Trapped peptides were eluted onto a reversed-phase PicoFrit column (New Objective, Woburn, MA) using a linear gradient of acetonitrile (0–60 %) containing 0.1 % formic acid. The duration of the gradient was 60 min at a flow rate of 0.25 nL/min. The eluted peptides from the PicoFrit column were sprayed into the LTQ XL equipped with a nanospray ion source. The data-dependent acquisition mode was enabled, and each survey MS scan was followed by five MS/MS scans with dynamic exclusion option on. The spray voltage and ion transfer tube temperatures were set at 1.8 kV and 180°C, respectively. The normalized collision energy was set at 35 %. The assay for each dietary treatment was run in three replicates.

For database searching and quantification, SEQUEST/Bioworks 3.3.1 SP1 was used to match MS/MS spectra to peptides using RefSeq database of *Drosophila melanogaster*, as described previously (Wu et al. 2007). Briefly, spectra/peptide matches were considered significant if the following criteria were met: a normalized difference in cross-correlation score (ΔC_n) of at least 0.08; minimum cross-correlation score (XC_{corr}) of 1.5 for +1, 2.0 for +2, and 2.5 for +3 charged ions; max XC_{corr} rank 1, and max Sp rank ≤ 10 . Search results from three replicates were merged into a single file per sample using Bioworks' multi-consensus function. The quantification of label-free samples was achieved using QUOIL label-free algorithm developed in house (Wang et al. 2006). Essentially, the label-free quantification was performed at the peptide level, where peptide identifications were assembled into a master list and a ratio of reconstructed chromatogram peak area between the contrasting samples for each identified peptide was calculated. The median of the computed peptide ratios represents the relative quantity of the protein in the two dietary treatments. The fold change induced by açai supplementation for each protein was calculated by dividing the number of identified peptides in the açai-treated group by that in the non-supplemented control. Each comparison was repeated three times.

Quantitative polymerase chain reaction

Three-day-old female *sod1*/RNAi flies were treated for 11 days on the SY diet supplemented or not with 2 % açai. Flies were then collected and frozen in liquid nitrogen. Fly heads were separated from bodies and collected using a sieve. Total RNA was isolated separately from fly heads or bodies using the Trizol reagent

from Invitrogen (Grand Island, NY) according to the manufacturer's suggested protocol. Quality and quantity of total RNA were assessed using the Nanodrop 1000 from Thermo Scientific (Wilmington, DE). cDNA was synthesized using Superscript[®] Reverse Transcriptase from Invitrogen. qPCR was performed using the StepOne real-time PCR System from Applied Biosystems (Grand Island, NY) according to the manufacturer's suggested protocol. Each qPCR measurement was repeated at least twice, each with four to six biologically independent samples. RpL32 was used to an internal control to normalize the transcript level of each gene.

Western blot analysis for 4-hydroxynonenal-protein adduct quantification

Protein was isolated from whole flies of 14-day-old fed diets supplemented with or without 2 % açai. Protein samples (20 µg/lane) were separated by electrophoresis in a 7 % NuPAGE Novex Tris–acetate SDS mini gel and transferred to a 0.2-µm polyvinylidene difluoride membrane. Membrane immobilized proteins were blocked and then incubated overnight at 4°C with anti-N α -acetyllysine-4-hydroxynonenal (4-HNE) fluorophore rabbit polyclonal antibody (Calbiochem, cat. no. 393206) to detect 4-HNE–protein adducts as described previously (Boyd et al. 2011). The blots were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG diluted 1:3,000 in the blocking solution for 90 min at room temperature. The immuno-reactive proteins were detected and quantified, as described previously (Boyd et al. 2011). At least three biological replicates were used for immunostaining. For 4-HNE-protein adduct measurement, the protein region between molecular weights 460 and 268 kDa was quantified. Relative 4-HNE-protein level was calculated by normalizing the 4-HNE-protein intensity value to the loading control β -actin.

Statistical analysis

All data were analyzed using StatView version 5.0 software (SAS, Cary, NC). For the life span data, Mantel-Cox logrank tests were performed by comparing flies fed the açai-supplemented diets to those fed the non-supplemented control diet. Student's *t* test analyses were performed for all other measurements. Statistical significance was set at $p < 0.05$. All life

span, food intake, locomotion, fecundity, qPCR, protein fold change and 4-HNE-protein adduct values are presented as means $\pm$ standard error.

Results

Açai supplementation at a later life stage promotes the survival of *sod1* knockdown flies

We previously showed that açai supplementation increases lifespan in *sod1*RNAi flies fed the standard SY diet (Sun et al. 2010). To determine the effect of açai supplementation that begins at a later life time we initiated açai supplementation for *sod1*RNAi female flies at 10 days of age. The age of 10 days is the time shortly before flies reach the stage when the mortality rate begins to accelerate based on the survival curve of *sod1*RNAi females (Sun et al. 2010). Açai supplementation at this later time still significantly extended lifespan in both *sod1*RNAi and a wild-type control line, UAS-*sod1*IR/+, when compared to the genotype-matched non-supplemented controls (Fig. 1). Açai supplementation increased mean lifespan in *sod1*RNAi females from 23.4 to 26.2 days for and in UAS-*sod1*IR/+ females from 31.5 to 37.0 days ($p < 0.001$). The mortality rate was significantly different between the supplemented and non-supplemented groups only after the initiation of açai supplementation for more than 10 days for *sod1*RNAi females (Fig. 1a). These findings indicate that it takes at least 10 days for the beneficial effect of açai supplementation on lifespan to manifest during a later life intervention.

Açai supplementation increased reproductive output in *sod1* knockdown flies

To determine the effect of açai supplementation on physiological function, we measured food intake and lifetime egg production of *sod1*RNAi and a wild-type control, da-Gal4/+, female flies. Consistent with previous results (Sun et al. 2010), we confirmed in this study that life-long açai supplementation extended lifespan of *sod1*RNAi flies (Fig. 2a). In addition, life-long açai supplementation also increased lifespan of the wild-type control da-Gal4/+ flies (Fig. 2b). We did not test the effect of life-long açai supplementation on lifespan in the other wild-type flies, UAS-*sod1*RNAi/+, since we already observed that even a later life

Fig. 1 The effect of a later life supplementation of açai on lifespan. **(a)** Lifespan curve of *sod1RNAi* (*UAS-sod1IR/+* and *da-Gal4/+*) female flies. **(b)** Lifespan curve of the wild-type control flies, *UAS-sod1IR/+*. Açai supplementation was

initiated at 10 days of age. Mean lifespan with standard error is listed in parentheses. *** $p < 0.001$ for comparison between açai-supplemented and genotype-matched non-supplemented groups by logrank test ($n = 133$ – 147 flies for each treatment)

intervention was sufficient to promote longevity in these flies as shown in Fig. 1b. We found that açai supplementation did not affect locomotor activity in *sod1RNAi* flies and their wild control flies when compared to the genotype-matched non-supplemented flies (data not shown). However, supplementation of açai at both 2 and 4 % increased lifetime egg production in *sod1RNAi* flies ($p < 0.05$), but not the wild-type *da-Gal4/+* flies, when compared to genotype-matched non-supplemented controls (Fig. 2c, d). We also assessed egg production during each interval of the times when flies were transferred to fresh food. The difference in egg production at each transfer emerged only after 14 days of açai supplementation, which was also the time when the mortality rate of non-supplemented *sod1RNAi* flies started to accelerate (Fig. 2e). Further, the duration of egg production was longer in açai-supplemented groups compared to the non-supplemented controls. These findings indicate that higher lifetime egg production in açai-supplemented flies was due to increased egg production in late life. The overall patterns of egg production were not significantly different in the wild-type *da-Gal4/+* flies treated with or without açai (Fig. 2f).

We measured food intake in flies fed the diet supplemented with or without 2 % açai. Açai supplementation at 2 % did not change daily food intake in both *sod1RNAi* and wild-type *da-Gal4/+* females (Fig. 2g, h). These findings suggest that food intake does not contribute to the differences in lifespan and reproductive output between *sod1RNAi* flies treated with and without açai.

The impact of açai supplementation on global protein expression

To investigate the molecular mechanisms by which açai supplementation promote survival in *sod1RNAi* flies, we measured changes in global protein expression induced by açai supplementation in fly bodies

Fig. 2 The effect of açai supplementation on physiological and behavioral function in *sod1RNAi* female flies and their wild-type control *da-Gal4/+* flies. **(a)** Lifespan curves of *sod1RNAi* females fed the 0, 2, and 4 % açai-supplemented diets. **(b)** Lifespan curves of the wild-type control *da-Gal4/+* females fed the 0, 2, and 4 % açai-supplemented diets. Mean lifespan with standard errors is listed in parentheses. *** $p < 0.001$ for comparison between açai-supplemented and non-supplemented groups by logrank test ($n \sim 30$ for each treatment). **(c)** Supplementation of 2 or 4 % açai significantly increased lifetime egg production when compared to the non-supplemented control in *sod1RNAi* flies. * $p < 0.05$; ** $p < 0.01$ by Student's *t* test; $n = 6$ replicates each with five females. **(d)** Supplementation of 2 or 4 % açai did not increase lifetime egg production relative to the non-supplemented control in the wild-type control *da-Gal4/+* flies ($n = 6$ replicates each with five females). **(e)** Egg production during each transfer interval per survived fly fed the 0, 2, or 4 % açai-supplemented diet throughout the adult life of *sod1RNAi* flies. Arrow indicates when the significant difference in egg production showed up between açai-supplemented and non-supplemented control flies. * $p < 0.05$ for comparison between açai-supplemented and non-supplemented groups by Student's *t* test ($n = 6$ for each treatment). **(f)** Egg production during each transfer interval per survived fly fed the 0, 2, or 4 % açai-supplemented diet throughout the adult life of *da-Gal4/+* flies. **(g)** Two percent açai supplementation did not change daily food intake in volume when compared to the non-supplemented control in *sod1RNAi* flies. **(h)** Two percent açai supplementation did not change daily food intake in volume when compared to the non-supplemented control for *da-Gal4/+* flies. a.u. refers to arbitrary unit

using a proteomic approach. Each comparison was repeated three times. After normalization, we identified 185 proteins with fold change values in all three replicates and 57 proteins with fold change values in two of the three replicates. From this set of proteins,

we found that açai supplementation induced significant changes in 22 proteins by >1.4-fold when compared to the non-supplemented control ($p < 0.05$; Fig. 3a, and Table 1). Among these 22 proteins, the expression levels of 20 proteins were decreased, while

Fig. 3 The effect of açai supplementation on changes in protein levels in *sod1RNAi* female flies. **(a)** The effect of açai supplementation on expression changes in 22 proteins. All fold changes in the graph are >1.4-fold and statistically significant at $p < 0.05$ by Student's *t* test ($n = 2-3$). **(b)** The changes at the transcript level for four genes, whose expression was altered at the protein level by açai supplementation shown in **(a)**. *l(1)*

G0156 refers to lethal (1) *G0156*, and *Zasp52* represents Z band alternatively spliced PDZ-motif protein 52. All transcript levels were normalized to the internal control Rpl32. *a.u.* refers to arbitrary unit. * $p < 0.05$; ** $p < 0.01$ for comparison between açai-supplemented and non-supplemented control groups by Student's *t* test ($n = 5-6$ for each treatment)

the levels of only two proteins, CG 6287 with phosphoglycerate dehydrogenase activity and ribosomal protein L12 (Rpl12 and CG3195), were increased by açai supplementation.

Based on the protein information in the flybase, we found that açai supplementation influence expression of proteins involving at least four biological processes

in *sod1RNAi* flies. First, açai supplementation decreased the protein levels of stress response genes. These include ferritin 2 light chain homologue (Fer2LCH, CG1469) and transferrin 1 (CG6186), involved in maintaining iron homeostasis (Heli et al. 2011; Xu et al. 2010); peroxiredoxin 5 (CG7217), an antioxidant (Radyuk et al. 2001); and turandot A (CG31509), a broad stress response

Table 1 The effect of açai supplementation on changes in protein levels

Gene symbol	Protein name	No. of replicates	Fold change ^a	<i>P</i> value ^b	Protein function ^c
CG33470	CG33470	2	-4.92±1.58	0.043	Novel
CG12233	Lethal (1) G0156	3	-4.22±2.06	0.023	Isocitrate dehydrogenase (NAD ⁺) activity
CG6186	Transferrin 1	3	-3.46±1.08	0.015	Iron ion transmembrane transporter
CG1469	Ferritin 2 light chain homologue	3	-3.28±0.34	<0.0001	Ferrous iron binding
CG32667	CG32667	2	-3.08±0.42	0.017	Novel
CG11963	skpA-associated protein	3	-3.01±1.25	0.04	Succinyl – CoA ligase
CG8050	Cystatin like	3	-2.44±0.51	0.028	Cysteine-type endopeptidase inhibitor
CG31509	Turandot A	2	-2.17±0.17	0.011	Response to various stresses, including bacterium, heat, and oxidative stress
CG15261	UK114	3	-2.15±0.32	0.007	Protein folding
CG42377	CG42377	3	-2.13±0.38	0.031	Novel
CG6783	Fatty acid-binding protein	3	-1.98±0.21	0.004	Fatty acid binding involved in long – term memory
CG8332	Ribosomal protein S15	3	-1.78±0.22	0.025	Structural constituent of ribosome involved in translation
CG7217	Peroxisredoxin 5	3	-1.77±0.17	0.007	Antioxidant
CG17903	Cytochrome c proximal	3	-1.71±0.16	0.011	Electron carrier in oxidative phosphorylation
CG3140	Adenylate kinase 2	3	-1.7±0.22	0.02	Adenylate kinase involved in neurogenesis
CG4696	Muscle protein 20	3	-1.65±0.18	0.012	Calcium ion bind activity involved in regulation of cell shape and myoblast fusion
CG4800	Translationally controlled tumor protein	3	-1.53±0.19	0.032	Guanyl – nucleotide exchange factor activity involved in positive regulation of cell size and multicellular
CG30084	Z band alternatively spliced PDZ organism growth motif protein 52, isoform K	3	-1.5±0.1	0.007	Protein binding activity involved in muscle structure development
CG8498	CG8498	3	-1.45±0.09	0.007	Fatty – acyl – CoA binding
CG12070	Saposin related	3	-1.41±0.09	0.009	dsRNA transport
CG6287	CG6287	3	1.78±0.06	0.005	Phosphoglycerate dehydrogenase
CG3195	Ribosomal protein L12	3	1.93±0.07	0.003	Structural constituent of ribosome involved in translation

^a Fold changes are expressed as mean ± standard error. "-" refers to proteins down – regulated by açai supplementation

^b The *p* values were from comparison between açai – treated flies and their non – supplemented controls by Student's *t* test

^c Protein function refers to known or prediction function based on the information from the flybase, www.flybase.org

protein (Zhang et al. 2011; Mahapatra et al. 2010). Second, açai supplementation affected the expression of proteins functioning in cellular respiration. Specifically, açai supplementation increased the expression of CG6287 with phosphoglycerate dehydrogenase activity, while decreasing the expression of cytochrome c proximal (CG17903), CG11015 with mitochondrial electron transport activity, skpA-associated protein (CG11963) with ATP-citrate lyase activity, and lethal (1) G0156 (CG12233) with isocitrate dehydrogenase activity.

Third, açai supplementation affected the expression proteins involving cellular growth. Açai supplementation increased the expression of RpL12, but decreased the expression of ribosomal protein S15 (CG8332). In addition, açai supplementation decreased the expression of muscle protein 20 (CG4696) and Z band alternatively spliced PDZ-motif protein 52 (Zasp52, CG30084) involved in muscle growth, fatty acid binding protein (CG6783) and adenylate kinase-2 (CG3140) involved in neurogenesis, and translationally controlled tumor

protein (CG4800) involved in regulation of cell size. Lastly, açai supplementation affects the expression of six other proteins, including saposin-related (CG12070) with double-stranded RNA binding activity, UK114 (CG15261) with endoribonuclease L activity, cystatin-like (CG8050) with cysteine-type endopeptidase inhibitor activity and three novel proteins, CG32667, CG33470, and CG42377. Together, these findings indicate that açai supplementation affects a broad range of biological processes involved in stress response and cellular and tissue homeostasis maintenance in *sod1RNAi* flies.

From the list of the 22 proteins significantly changed by açai supplementation, we selected four candidate genes to measure their transcript levels in bodies of açai treated flies and the non-supplemented controls. Two out of these four genes, *transferrin 1* (CG6186) and *turandot A* (CG31509), displayed decreases at the transcript level by açai supplementation, which were similar to changes in the protein expression. However, two genes, *l(1)G0156* and *Zasp52*, showed no change at the transcript level (Fig. 2b). These findings indicate that changes at the transcript and protein levels induced by açai supplementation are not necessarily the same.

Açai supplementation influences expression of longevity related genes

To further investigate the molecular mechanisms underlying açai-induced pro-survival, we measured transcript changes in genes associated with oxidative stress, longevity, and metabolic pathways for 14-day-old *sod1RNAi* female flies fed the SY diet with or without 2 % açai. The identities of these genes were described previously (Boyd et al. 2011). Further, the expression of these genes was measured separately in fly heads and bodies to determine body part-specific expression patterns. The relative transcript levels of a subset of these genes that had significant changes in fly heads are shown in Fig. 4a and the expression patterns of the same genes in fly bodies are shown in Fig. 4b.

We found that açai supplementation affected at least three classes of genes. First, açai supplementation reduced the transcript levels of several downstream targets of the JNK pathway, including *lethal (2) essential for life* (*l(2)efl*), *glutathione S-transferase D1* (*gstD1*), and *growth arrest and DNA-damage inducible 45* (*Gadd45*), in the heads of flies fed 2 % açai compared to the non-

supplemented controls (Fig. 4a, $p < 0.05$, Jasper et al. 2001; Wang et al. 2003, 2005). Antioxidant gene peroxiredoxin 2540, a member of peroxiredoxin family involved in detoxification (Wood et al. 2003), was decreased by açai supplementation. Second, we observed that two genes involved in mitochondrial biogenesis, *mitochondrial assembly regulatory factor* (*Mfn2*) and *nitric oxide synthase* (*NOS*), were up-regulated by açai supplementation in fly heads fed the açai-supplemented diet relative to the non-supplemented controls (Civitarese et al. 2007). Açai supplementation also increased the expression of *target-of-rapamycin* (*TOR*) involved in protein metabolism, autophagy and mitochondrial biogenesis (Zoncu et al. 2011; Kim and Guan 2011). Third, a significant decrease at the transcript level of *phosphoenolpyruvate carboxykinase* (*PEPCK*), a gluconeogenesis gene, was observed in the heads of flies fed 2 % açai relative to the non-supplemented controls. Most of the genes tested showed similar patterns of açai-induced changes between fly heads and bodies (Fig. 4a, b). However, some downstream targets of the JNK signaling, including *l(2)efl* and *GstD1*, were altered in fly heads but not bodies (Fig. 4b). These findings indicate that açai supplementation modulates expression of some genes in a body part-specific manner.

Açai supplementation reduces oxidative damage

To directly examine the effect of açai supplementation on oxidative damage, we measured the level of 4-HNE-protein adducts, a lipid oxidation marker (Ferrara et al. 2008; Kohen and Nyska 2002), in whole bodies of 14-day-old *sod1RNAi* female flies fed the SY diet and the SY diet supplemented with 2 % açai. We found that supplementation of 2 % açai significantly reduced the level of 4-HNE-protein adducts in cytosolic protein of whole flies by approximately 40 % compared to the non-supplemented control ($p < 0.001$, Fig. 5). These findings support the notion that açai supplementation can reduce oxidative damage in *sod1RNAi* flies.

Discussion

Numerous studies link oxidative damage to aging and degenerative diseases (Perez et al. 2009; Salmon et al. 2010). Interventions using nutraceuticals are promising

Fig. 4 Gene expression changes induced by açai supplementation in *sod1RNAi* female flies. **(a)** The transcript levels of eight genes in fly heads from non-supplemented and 2 % açai-supplemented treatments. **(b)** The transcript levels of the same eight genes in fly bodies from non-supplemented and 2 % açai-supplemented treatments. The full names of all genes are described

in the main text. The transcript levels of all genes were normalized to the internal control RpL32. Error bars refer to standard errors. a.u. refers to arbitrary unit. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for comparison between açai-supplemented and non-supplemented control groups by Student's *t* test ($n = 5-6$ for each treatment)

Fig. 5 The effect of açai supplementation on lipid oxidation in *sod1RNAi* female flies. The levels of 4-hydroxynonenal (4-HNE)-protein adducts were detected by Western blot and semi-quantified in protein samples from whole flies fed the diets supplemented or not with 2 % açai. β -actin was used as a loading control. Western blot images from three biological

replicates are shown on the *left panel*. The 4-HNE/actin ratios were calculated from five biological replicates for each treatment. *a.u.* refers to arbitrary unit. *** $p < 0.001$ for comparison between açai-supplemented and non-supplemented control groups by Student's *t* test

approaches to delay the onset of aging and age-related diseases, such as diabetes and cardiovascular and degenerative diseases (Joseph et al. 2007). However, the optimal intervention protocols using nutraceuticals and the molecular mechanisms underlying the health benefits of nutraceuticals remain elusive. We previously demonstrated that a botanical made from açai pulp extract promotes the survival of flies fed a high-fat diet or *sod1RNAi* flies fed a standard diet (Sun et al. 2010). Here, we extended our previous study to investigate the physiological and longevity effects of açai supplementation in *sod1RNAi* flies and evaluated underlying molecular mechanisms. We confirmed that life-long supplementation of açai promotes survival in *sod1RNAi* flies. We further demonstrated that açai supplementation initiated at a later life stage was sufficient to extend the lifespan of *sod1RNAi* flies and their wild-type control flies. The lifespan extension was associated with an increase of lifetime reproductive output induced by açai supplementation in *sod1RNAi* flies but not their wild-type control flies. Our mechanistic studies indicate that açai supplementation affected the protein and/or transcript levels of genes involved in oxidative stress response, mitochondrial biogenesis, translation, and nutrient metabolism in *sod1RNAi* flies. Further, the açai-induced lifespan extension was associated with a decrease of 4-HNE-protein adducts, a lipid oxidation marker, in *sod1RNAi* flies. Together, our findings suggest that açai pulp can be a potent nutraceutical to promote healthy aging in *sod1-*

deficient flies partly through modulating nutrient metabolism and reducing oxidative damage.

Our previous studies indicated that açai pulp promotes the survival and modulates the transcript levels of several downstream targets of JNK signaling, including *l(2)efl* and *GstD1*, in wild-type flies fed a high-fat diet (Sun et al. 2010). JNK signaling is a major pathway responding to oxidative stress and has been implicated in aging and aging-related diseases (Karpac and Jasper 2009). Our previous findings suggest that açai extends lifespan in flies fed a high-fat diet partly through activation of JNK signaling and alleviation of oxidative stress (Sun et al. 2010). We also showed that açai pulp improves survival in *sod1RNAi* flies fed a standard diet. However, the mechanisms underlying the beneficial effect of açai to *sod1RNAi* flies have not been investigated. In this study, we addressed these mechanistic issues by measuring the transcript changes in a number of longevity-related genes, global expression changes at the protein level and the lipid oxidation level in *sod1RNAi* flies. We found that the transcript levels of *l(2)efl* and *GstD1*, two JNK signaling targets, were decreased in the head or unchanged in the body by açai in *sod1RNAi* flies fed the standard diet. These two genes, however, are up-regulated by açai in wild-type flies on the high-fat diet (Sun et al. 2010). Another JNK signaling target *Gadd45*, not investigated in high-fat fed flies, was also down-regulated by açai in both

heads and bodies of *sod1RNAi* flies. The relationship between JNK signaling and lifespan is complex (Karpac and Jasper 2009). On the one hand, modest activation of JNK signaling has been shown to extend lifespan in worms and flies. On the other hand, deficiency or over-activation of JNK signaling is detrimental to longevity. One possible explanation of the contradicting findings in wild-type and *sod1RNAi* flies is that the effect of açai on JNK signaling depends on the redox status of flies. Further studies are required to investigate these dichotomous effects of açai on JNK signaling.

Our expression studies reveal that açai supplementation decreases expression of several genes involved in oxidative stress response. We found that the transcript level of Prx2540 and the protein levels of Prx5, Fer2LCH, and transferrin 1 were significantly lower in açai-supplemented *sod1RNAi* flies. In addition, açai supplementation significantly reduced the level of 4-HNE-protein adducts, a lipid oxidation marker in *sod1RNAi* flies (Ferrara et al. 2008; Kohen and Nyska 2002). Part of these findings is consistent with some of our previous results indicating that the transcript level of Prx2540 is decreased by açai in wild-type flies fed the high-fat diet. Peroxidoxins are a family of antioxidant enzymes that are induced under oxidative stress and are involved in detoxification (Wood et al. 2003; Zimniak 2008), Fer2LCH and transferrin 1 involve iron ion homeostasis, which is known to be critical in oxidative stress response (Heli et al. 2011; Xu et al. 2010). These findings along with our observation from studying the downstream genes of JNK signaling suggest that açai reduces oxidative damage probably through its antioxidant activity, which, in turn, indirectly reduces the expression of oxidative stress response genes.

An increasing body of evidence indicates that some nutraceuticals are potent in modulating nutrient metabolism, such as glucose metabolism (Bao and Fenwick 2004; Obrenovich et al. 2010). Consequently, nutraceuticals have been proposed to be efficient agents for preventing metabolic diseases, such as diabetes and atherosclerosis, and delaying degenerative diseases. Nutraceuticals including blueberry, cranberry, and broccoli extracts have been shown to delay the age-related decline of cognitive function in rodent models (Joseph et al. 2007, 2009). Several studies have shown that ALS patients may benefit from taking a variety of fruits and vegetables and consuming a

hypocaloric diet (Magnuson et al. 2011; Okamoto et al. 2009). In this study, we found that açai supplementation decreased the transcript level of PEPCK, and the expression levels of several mitochondrial proteins with ATP-citrate lyase and isocitrate dehydrogenase activities in *sod1RNAi* flies. This is consistent with our previous finding that açai supplementation decreases the transcript level of PEPCK in wild-type flies fed the high-fat diet. PEPCK is a key enzyme involved in gluconeogenesis and modulates blood glucose levels (Chakravarty et al. 2005). Inhibition of PEPCK is a therapeutic approach to control symptoms of high glucose levels in diabetic patients (Srivastava 2009). A pilot human study revealed that consumption of açai pulp reduces the levels of several risk markers of metabolic disease in overweight adults, including fasting glucose and insulin, total cholesterol and post-prandial plasma glucose (Udani et al. 2011). In addition, we have found that açai supplementation increased the transcript level of TOR. Reduction of the TOR signaling, an amino acid-sensing pathway, has been shown to promote longevity through decreasing translation and increasing mitochondrial biogenesis and autophagy (Zoncu et al. 2011; Kim and Guan 2011). However, açai supplementation increases the transcript levels of Mfn2 and NOS in *sod1RNAi* flies, which promote mitochondrial biogenesis (Civitarese et al. 2007). One possible scenario is that açai may reduce the activity of TOR signaling, which in turn increases the TOR transcript level through a feedback mechanism. Together, our findings suggest that açai supplementation promotes healthy aging through improving glucose metabolism and mitochondrial biogenesis in *sod1RNAi* flies. Further studies are required to determine the effect of açai supplementation on biochemical and cellular and metabolic functions of flies.

Based on typical lifespan curves, life of adult flies and other animals can be generally divided into three stages: young, middle, and old age. In this study, we found that the pro-survival effect of açai supplementation in *sod1RNAi* flies does not require life-long intervention. Açai supplementation was effective in promoting survival even when initiated in *sod1RNAi* flies entering in their middle-age stage. We found that açai-induced increase of reproductive output begins to manifest in middle age of *sod1RNAi* flies. This finding places açai in a similar category to a growing number of nutraceuticals and polyphenolic compounds that can

promote health benefits even when supplementation is not initiated in early adulthood of animals. To name a few, a botanical containing oregano and cranberry extracts is sufficient to extend lifespan in Mexican fruit flies when supplemented in the middle age (Zou et al. 2010, 2011). Resveratrol, a polyphenol, has been shown to extend lifespan of middle-aged mice on a high-fat diet (Baur et al. 2006). Rapamycin, an inhibitor of TOR, can extend lifespan in middle-aged mice (Harrison et al. 2009). It will be interesting to further determine in which of the three life stages açai supplementation is still sufficient to improve the health of animals and patients with high oxidative stress levels and investigate the underlying mechanisms.

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