Available online at www.sciencedirect.com
ScienceDirect
Pharmacology, Biochemistry and Behavior 85 (2006) 620–628
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Beneficial effects of natural antioxidants EGCG and α-lipoic acid on life span and age-dependent behavioral declines in *Caenorhabditis elegans*

Marishka K. Brown [a], Joseph L. Evans [b], Yuan Luo [a,*]

[a] *Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 N. Pine St, PH501, Baltimore, MD 21201, USA*
[b] *Medical Research Institute, San Francisco, CA, 94107, USA*

Received 26 June 2006; received in revised form 6 October 2006; accepted 25 October 2006
Available online 5 December 2006

Abstract

Oxidative stress has been associated with both the aging process and the development of age-dependent tissue degenerative pathologies. Beneficial effects of antioxidant therapies to abrogate the deleterious consequences of elevated free radicals are implicated in disease prevention and cost-effective strategy. Previous data have shown protective effects of the polyphenol green tea constituent epigallocatechin gallate (EGCG) and a classic natural antioxidant α-lipoic acid (LA) against oxidative stress and aging. In this study, EGCG and α-lipoic acid were applied to model *Caenorhabditis elegans*, and their ability to modulate the life span and several age-associated behavioral declines were examined, including: pharyngeal pumping, chemotaxic behavior and amyloid β-associated pathological behavior. It was demonstrated that both antioxidants attenuated the levels of hydrogen peroxide in *C. elegans*, but their effects on age-dependent decline in behaviors were different. EGCG, but not α-lipoic acid, attenuated the rate of decline in pharyngeal pumping behavior in *C. elegans*. In contrast, α-lipoic acid, but not EGCG, extended mean and maximal life span in *C. elegans*. Both EGCG and α-lipoic acid were able to facilitate the chemotaxis index and this effect was additive. Furthermore, EGCG, but not α-lipoic acid, moderately alleviated an Aβ-induced pathological behavior in a transgenic *C. elegans* strain. These results indicate that natural antioxidants can protect against age-dependent behavioral declines. Other protective mechanisms, in addition to their antioxidant properties, may underlie their differential beneficial effects on aging and physiological behaviors.
© 2006 Elsevier Inc. All rights reserved.

Keywords: EGCG; α-lipoic acid; Animal behaviors; Aging; Antioxidants

1. Introduction

The use of herbal products and dietary supplements has dramatically increased in the US over the past 10 years (Eisenberg et al., 1998) with the majority of dietary supplements being marketed as possessing antioxidant properties. The mechanism of these supplements in recent scientific studies has garnered attention because of the beneficial effects and their low instances of cell toxicity, compared to most of the synthetic drugs. However, the difference between these compounds affecting defined physiological and pathological behaviors has not been evaluated *in vivo*.

Among more than 2000 dietary supplements on the market, epigallocatechin-3-gallate (EGCG) and α-lipoic acid (LA) are of particular interest because of their reported protective properties against oxidative stress (Packer et al., 1995; Higdon and Frei, 2003). Green tea is rich in catechins, the most active and abundant being EGCG, which has been demonstrated to have antimutagenic effects *in vivo* (Muto et al., 1999) as well as being a potent neuroprotective agent (Jin et al., 2001). EGCG has several biological and pharmacological properties that include: free radical scavenging activity, antioxidant actions, iron-chelating capabilities and attenuation of lipid peroxidation due to various forms of radicals (Guo et al., 1996). EGCG has also been shown to protect and rescue PC12 cells against amyloid β-induced neurotoxicity in a dose dependent manner (Levites et al., 2003; Bastianetto et al., 2006).

The natural antioxidant, thioctic acid, more commonly known as α-lipoic acid (LA), exhibits effects on increasing insulin sensitivity when administered orally in patients diagnosed with type-2 diabetes (Jacob et al., 1999). LA is synthesized in

* Corresponding author. Tel.: +1 410 706 6639; fax: +1 410 706 4376.
E-mail address: yluo@rx.umaryland.edu (Y. Luo).

0091-3057/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2006.10.017

organisms ranging from bacteria to humans and is a natural cofactor in the pyruvate dehydrogenase complex where it binds acyl groups and transfers them from one complex to another (Biewenga et al., 1997). The only natural form of LA is the *R*-stereoisomer, which is a minor component of certain foods (Packer et al., 1995). Thus far, LA and its reduced form, dihydrolipoic acid (DHLA), have demonstrated three distinct antioxidant actions. Both LA and DHLA have metal-chelating properties along with the ability to scavenge harmful reactive oxygen species (Biewenga et al., 1997). LA exhibits networking capabilities, because it has the capacity to regenerate endogenous antioxidants (Biewenga et al., 1997). LA has also been reported to enhance learning in old mice (Farr et al., 2003) and extending life span in the fruit fly *Drosophila melanogaster* (Bauer et al., 2004).

Aging is the progressive accumulation of changes over time that is associated with or responsible for the increasing susceptibility to disease and death (Harman, 1981). Although there are several theories that are though to contribute to the aging process, the free radical theory of aging seems to be a significant contributor in the process. Free radicals are highly reactive by-products of metabolism seeking stability by gaining an extra electron from any source. This very property leads to protein, lipid, and DNA damage.

Once produced, free radicals are normally cleared by the body's naturally occurring antioxidants, but as organisms age, the declining efficiency of this mechanism allows for the accumulation of free radicals in the body (Harman, 1956). Evidence provided by previous research has correlated neural activity with the aging process (Wolkow, 2002). The development of neurodegenerative pathologies such as Alzheimer's disease (AD), a multifaceted disease with risk factors that include: gene mutation, diet, environment, and oxidative stress, have added another dimension to the inevitable aging process.

For years, caloric restriction was the only proven method shown to increase the life span of experimental animals. More recently, life span increase due to several antioxidants, e.g. resveratrol and blueberry extract were reported in simple animal models such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *D. melanogaster* (Bauer et al., 2004; Wood et al., 2004; Wilson et al., 2006). In one of our previous studies, we demonstrated that EGb 761, an extract derived from the leaves of the *Ginkgo biloba* tree, increased the life span and oxidative stress resistance in *C. elegans* (Wu et al., 2002). These results indicate that life span modulation may be achieved pharmacologically, and may be partially mediated through antioxidant mechanisms.

The soil nematode *C. elegans* is known as a model for studying the molecular mechanisms of the aging process (Luo, 2004). More than 50 genes have been identified that alter gene activities ranging from increased longevity to accelerated aging (Hekimi and Guarente, 2003). The worms exhibit many behavioral phenotypes including egg laying, locomotion, chemotaxis, stress response and associative learning etc., which are well employed in developmental biology, genetics and neurobiology studies.

The present study aimed to determine if EGCG and LA share similar pathways to reduce oxidative stress and modulate age-associated behavioral declines in the model organism *C. elegans*. First, the effect of EGCG and LA on the levels of endogenous hydrogen peroxide (H_2O_2) and on the animal's life span in both the wild type N2 *C. elegans* and *daf-16 (mgDf50)*, which is defective in mediating insulin signaling pathway (Ogg et al., 1997) were compared. Then, the effect of EGCG and LA on the performance of two characteristic *C. elegans* behaviors; pharyngeal pumping and chemotaxis were examined. Furthermore, a pathological behavior phenotype due to the expression of amyloid β in *C. elegans* was also explored.

2. Materials and methods

2.1. Reagents

Epigallocatechin-3-gallate (EGCG), a polyphenol compound was purchased from Sigma (St. Louis, MO). Stock solutions of EGCG were made in 100% ethanol. Final concentrations of EGCG ranged from 10 μM to 100 μM. The final concentration for ethanol was less than 0.1% when dissolved in the food source of *C. elegans*.

α-lipoic acid (LA) was obtained from Medical Research Institute (San Francisco, CA), who purchased it from Antibioticos SA (Rodano, Italy). This form is a racemic mixture composed of both the *R*- and *S*-enantiomers of LA present in approximately a 1:1 ratio. It is of the highest purity commercially available (≥98.0% purity). Although high-purity racemic LA is available from Sigma and other biochemical reagent suppliers, MRI purchases LA from Antibioticos because of its availability in metric ton quantities (for commercial use as a dietary supplement), and is of the highest purity on the commercial market. LA was dissolved in 100% ethanol to make the stock solution. The final concentrations of LA ranged from 10 μM to 100 μM. Most of other reagents were purchased from Sigma (St. Louis, MO).

2.2. Strains of C. elegans

Experimental *C. elegans* were wild type N2 (Bristol), transgenic mutant strain *daf-16 (mgDf50)*, and the transgenic Aβ muscle expressing strain CL4176. The wild type N2 strain was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). The *daf-16 (mgDf50)* mutant strain was provided by Dr. C. Wolkow of NIH/NIA. The *daf-16* phenotype has a slightly decreased life span in comparison to N2 and is heat sensitive. The strain CL4176 was provided by Dr. C. Link of the University of Colorado. CL4176 is a temperature-sensitive mutant strain that expresses human Aβ1-42 only when it reaches non-permissive temperatures. The expression of Aβ in muscle cells causes paralysis in these mutants (Link et al., 2003).

2.3. Behavioral experiments

Most of the behavior experiments were performed on solid nematode growth medium (NGM), with a 100 μl spot of *Escherichia coli* OP50 for food (Brenner, 1974). Reproductive adults were transferred onto fresh NGM plates and allowed to

lay eggs for 2–4 h, producing age-synchronized groups of worms. All worms were cultivated at 20 °C in a temperature-controlled incubator. The chemicals were added directly to the OP50 food source to feed the worms.

2.4. DCF assay of H_2O_2 levels

The assay was conducted as described previously (Smith and Luo, 2003). 4-day old age synchronized worms were collected in 100 μl of 0.1% PBST. The worms were then homogenized with a sonicator. 5 μl of the fluorescent dye dichlorofluorescin diacetate (DCF-DA) was added to 10 ml of PBST. 100 μl of this dye mixture was then added to the sonicated worms. 200 μl of each sample were transferred into a 96 well plate and placed into a fluorescent microplate reader for 2 h and 30 min, set to measure fluorescence levels at 10 min intervals. For quantification, fluorescence excitation was read at 485 nm and emission read at 528 nm. For statistical analysis, the endpoint readings upon completion of the cycle were used.

2.5. Life span assay

As described previously (Wu et al., 2002), age synchronized young larvae (L1) were fed with different agents and maintained at 20 °C. The worms were then transferred to fresh plates on the 4th day after hatching. Once the worms reached adulthood (L4), they were transferred daily for 6 consecutive days until the cessation of egg laying to avoid overlapping generations. After these 6 consecutive days, the worms were transferred every other day. Worms were scored as dead if they did not respond to touch stimulus.

2.6. A, B, C mobility assay

N2 worms were treated from L1 until completion of the assay. 100 worms in each treatment group were assayed. Worms were scored as A, if they moved spontaneously and without the aid of touch stimulus. Worms were scored B, if a touch stimulus was needed in order for the worms to move. Worms were scored as C, if they only moved their head or tail and it required a touch stimulus.

2.7. Pharyngeal pumping assay

Worms were treated with EGCG or LA from day one young larvae (L1) until death. Pharyngeal pumping was recorded everyday along with life span from adult day 1 until death of the worms. The pumping assays were performed on NGM agar plates at room temperature. A Nikon SMZ1000 stereoscopic zoom microscope was used for the assay. Pharyngeal pumping was defined as the number of times the terminal bulb of the pharynx contracted over a one-minute interval. All pumping rates were measured on a lawn of OP50 bacteria.

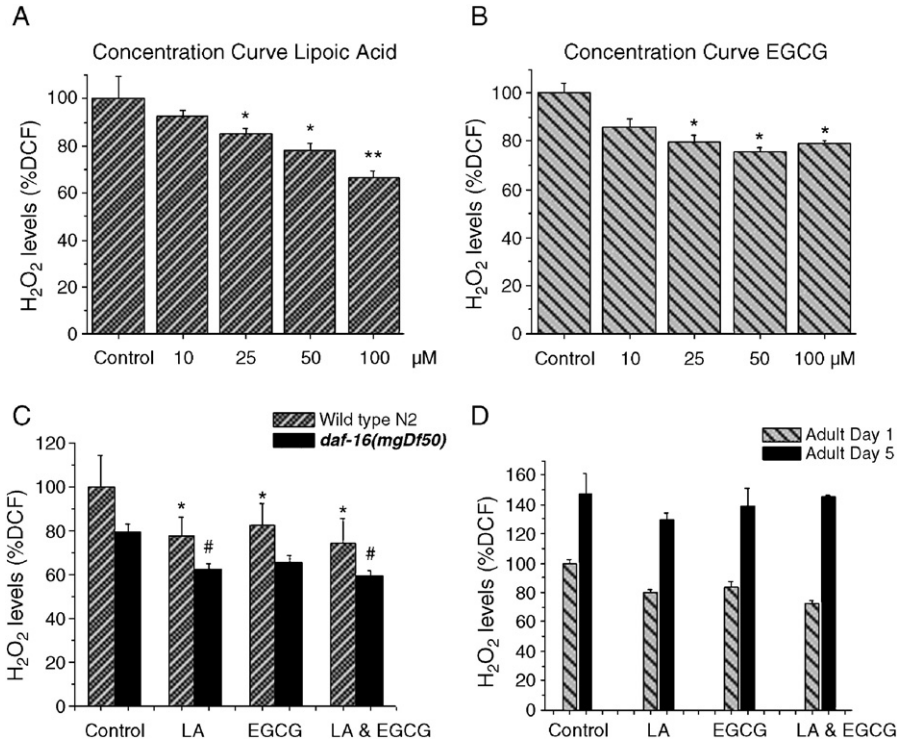

Fig. 1. Levels of H_2O_2 in the worms fed with different concentrations of LA (A) or EGCG (B). *C. elegans* were grown on solid NGM media at 20 °C. 10–100 μM concentrations of LA (A) or EGCG (B) were added directly to OP50 food source and animals were treated from L1 until experimentation. Following treatment, age synchronized 4 days old (L4) nematodes were collected and incubated with DCF-DA (see Methods). Levels of H_2O_2 were detected at 37 °C in a fluorescent microplate reader. Data are expressed as percentage change of the levels of H_2O_2 compared with untreated control as 100%. C. Levels of H_2O_2 in wild type (dashed bars) or a *daf-16 (mgDf50)* mutant (black bars) *C. elegans* fed with or without natural antioxidants. D. Levels of H_2O_2 in adult day 1 worms (dashed bars) compared with that in adult day 5 worms (black bars). Each graph represents at least 3 independent experiments with at least 100 worms in each treatment group.

2.8. Chemotaxis assay

Chemotaxis assays were performed as described by Bargmann et al. (1993) at room temperature, which is approximately 23 °C. Adult day 1 and adult day 5 worms, from the same population, were used for these experiments. Worms were treated from young larvae (L1) until they were assayed. The worms were briefly washed in M9 buffer, and 20–30 worms were placed on 10 ml of solid assay agar plates containing 1 μl of attractant placed on the edge of each plate. After all animals were transferred to the center of the assay plate, a second drop of attractant, diacetyl 1:100 vol/vol, along with 1 μl of 1 M sodium azide was added to the original spot. On the opposite side of the attractant, 1 μl drop of sodium azide was added to paralyze the worms if they ventured in the opposite direction of the attractant. Worms were counted following an elapsed time period of 1 h. Chemotaxis was quantified by a chemotaxis index, calculated as the number of animals that arrived at the attractant location, i.e., chemotaxis index = (number of worms at the attractant location − number of worms at the control location)/total number of worms on the plate. All experiments were performed with three independent populations.

2.9. Aβ-induced paralysis assay

The paralysis assay of transgenic strain CL4176 fed with drugs was previously described (Gutierrez-Zepeda et al., 2005). To prepare age-synchronized transgenic *C. elegans* (CL4176), nematodes were transferred to fresh NGM plates upon reaching reproductive maturity at 3 days of age and allowed to lay eggs overnight. Isolated hatchlings (100 each group) from the synchronized eggs (day 1) were cultured on fresh NGM plates in a 16 °C temperature-controlled incubator (Sheldon Manufacturing, Model 2005, Cornelius, OR). The worms were fed with the drugs starting from the egg. Temperature up-shift was started at the 36th hour after hatching by up-shifting the temperature from 16 °C to 23 °C to induce Aβ transgene expression, and lasted until the end of the paralysis assay. Paralysis was scored at 30 min intervals until the last worm demonstrated paralysis.

2.10. Statistical analysis

Statistical analyses were performed using either a one-way ANOVA or Student's *t* test in Microsoft Origin Software (Northampton, MA) to compare control and treated groups with *p*-value equal to or <0.05 being considered statistically significant.

3. Results

3.1. Both EGCG and α-lipoic acid diminishes free radical H_2O_2 levels in C. elegans

Before comparing the effects of EGCG and LA on age-dependent decline of physiological and pathological behaviors in the *C. elegans,* their antioxidative potencies in *C. elegans* were first determined using a DCF method established previously (Smith and Luo, 2003). Age-synchronized worms were exposed to different concentrations of EGCG and LA, which ranged from 10 μM–100 μM in OP50 bacteria food lawn. A group of 100 worms were fed either with vehicle control or one of the following compounds, EGCG or LA at 10, 25, 50 or 100 μM or their combination starting at L1 (Fig. 1A and B). At the first day of adulthood (L4), the age-synchronized worms were collected for DCF assay of the levels of H_2O_2 using a fluorescent microplate reader. For this assay, three independent

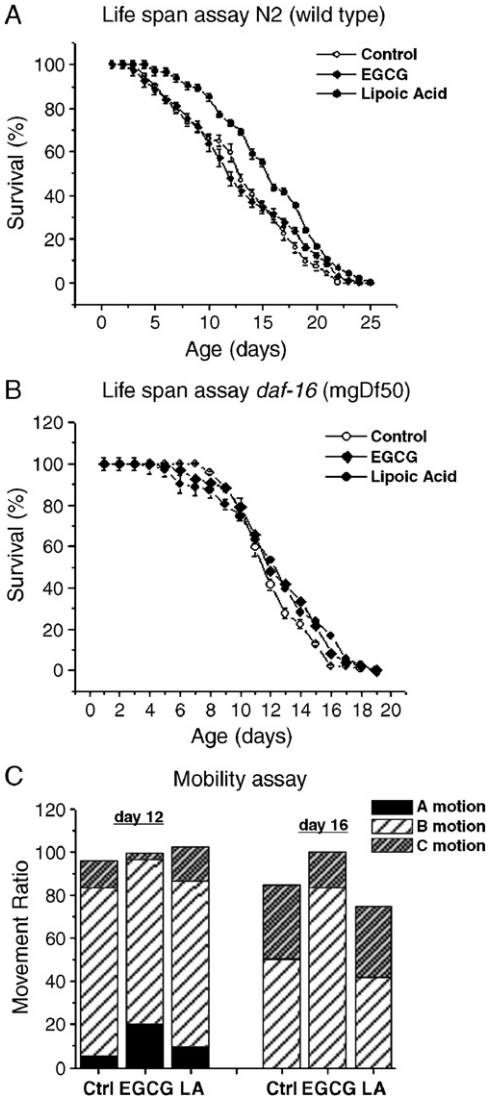

Fig. 2. Effects of LA and EGCG on life span of wild type and *daf-16 (mgDf50) C. elegans*. The wild type N2 (A) or *daf-16 (mgDf50)* (B) worms were placed on plates that had no drugs (open circles), or were seeded in OP50 containing 25 μM EGCG (closed diamonds) or 24 μM LA (closed circles) from day one of life span. Worms were transferred to fresh plates starting from L4 for four consecutive days, then every other day. Survival rate was scored everyday and is expressed as percentage of survival. At least 3 independent assays in each graph and 100 worms in each assay group. C. Locomotive assay in wild type *C. elegans* fed with EGCG or LA at day 12 or day 16 of age. N2 worms were treated with 25 μM EGCG or 24 μM LA from L1 until completion of the assay. Worms were scored as A (filled bars), B (dashed bars) or C (dark dashed bars) defined in Methods. Motility rate is expressed as percentage compared with untreated control set as 100%.

Table1
Summary of life span assay

Genotype	Drug treatment (μM)	Mean lifespan±SD (days)	*p*-value	Maximum lifespan±SD (days)	*p*-value
Wild type (N2)	Ctrl	12.4±0.5		21±1.1	
	EGCG (25)	13.0±1.6	0.58	22±2.0	0.07
	Lipoic acid (24)	15.4±1.0	0.01**	24±1.0	0.04*
daf-16 (mgDf50)	Ctrl	11.7±1.1		18±1.5	
	EGCG (25)	12.7±0.3	0.19	18±0.6	0.35
	Lipoic acid (24)	12.5±0.1	0.24	18±1.1	0.58

The effect of different drug treatments on the life span of the wild type (WT) strain as well as *daf-16 (mgDf50) C .elegans* was determined. The life span assays were all performed at 20 °C. Worms were treated with either 24 μM LA or 25 μM EGCG from L1 until the end of the assay. The worms were transferred to a new plate with OP50 every other day for the first 6 days (see Methods). The mean life span was calculated as the average number of days by which the worms survived in an assay population. At least three assays were performed for each population. The total number of worms for each assay is 100. *$p<0.05$, **$p<0.01$.

experiments were performed, with the total number of worms equaling 150. Dose-dependent attenuation of H_2O_2 levels in the wild type worms fed with LA is shown in Fig. 1A. Similar results were obtained with EGCG (Fig. 1B), and when comparing LA and EGCG at the same concentration (25 μM; Fig. 1C). Significant differences were observed for both treatments, and the combination, compared with the vehicle-treated control wild type worms (dashed bars, EGCG $p=0.045$, LA, $p=0.013$; EGCG+LA, $p=0.015$). The additive effects were not significant compared with each drug treatment alone in the wild type worms (dashed bars). The intracellular H_2O_2 levels of the *daf-16 (mgDf50)* worms fed with both agents were also measured because of its increased sensitivity to oxidative stress (Yanase et al., 2002). Attenuation of H_2O_2 levels by 25 μM EGCG in the mutant strain was not significantly different compared with the untreated controls (black bars, $p=0.145$). However, feeding with LA and a combination of LA and EGCG showed statistically significant effects in *daf-16 (mgDf50)* ($p=0.002$ and 0.001 respectively, Fig. 1C). Interestingly, at an older age (adult day 5), there was a significant increase in levels of H_2O_2 (Control adult days 1 and 5; $p=0.02$), but neither one of the drugs attenuated the enhanced H_2O_2 levels in the wild type worms at this age (Fig. 1D). The basal levels of H_2O_2 in *daf-16 (mgDf50)* worms is significantly lower than in the wild type controls (Fig. 1C, $p=0.02$).

3.2. Lipoic acid extends the life span of C. elegans

To determine whether EGCG or LA would affect the maximal life span of *C. elegans*, typically about 20 days at 22 °C, a life span assay was conducted. Synchronized worms were fed with 25 μM of EGCG or 24 μM of LA and their life span was measured. In three independent experiments, with the number of worms totaling 100 for each treatment, there was a significant difference in the mean and maximal life span in worms fed with LA, but not EGCG, as shown in Fig. 2A and Table 1. To determine a possible mechanism for this life span extension by LA, a life span assay in the mutant worm *daf-16 (mgDf50)* was performed. DAF-16 is a down stream suppressor for insulin receptor cell signaling. The worm *daf-16 (mgDf50)* exhibits a shortened life span and increased sensitivity to oxidative stress (Yanase et al., 2002). If the *daf-16*-encoded protein mediates the effect of LA, the effect of LA on life span extension would be diminished in *daf-16 (mgDf50)* that was fed LA. As shown in Fig. 2B, the survival curve did not exhibit significant difference among worms fed with LA or EGCG, suggesting that extension of life span by LA is dependent on the expression of DAF-16. Table 1 summarizes the results of the life span assays. Among wild type worms fed with EGCG and LA, only LA significantly extended the mean and maximal life span. This significant extension of life span is not shown in *daf-16 (mgDf50)* (Table 1).

Although EGCG did not have any significant effects on the mean or maximum life span of the worms, there was a

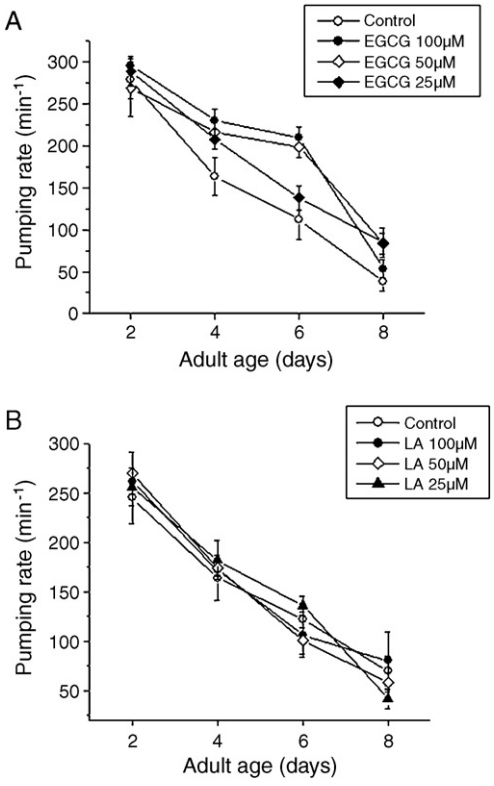

Fig. 3. Pharyngeal pumping assay in *C. elegans*. N2 worms were treated with 10–100 μM EGCG (A) or 10–100 μM LA (B) starting from day 1 young larvae (L1). Pharyngeal pumps were scored everyday from adult day 1 until the end of their life span to assay functional decline of the pharynx muscle ($n=12$ animals for each group). The rates of decline of the muscle contractions in the animals fed with higher concentrations (50 μM and 100μM) of EGCG were significantly attenuated. B. Varying concentrations of LA had no statistically significant effect on pharyngeal pumping in *C. elegans*. Only adult day 2 through adult day 8 are depicted in the graph for clarification.

Table 2
Summary of pharyngeal pumping assays

Drug (μM)	Day 2 *p*-values	Day 4 *p*-values	Day 6 *p*-values	Day 8 *p*-values
Ctrl.				
EGCG (100)	0.49	0.02*	0.003**	0.37
EGCG (50)	0.82	0.18	0.004**	0.03*
EGCG (25)	0.72	0.13	0.43	0.04*
EGCG (10)	0.33	0.16	0.06	0.51
LA (100)	0.58	0.82	0.56	0.74
LA (50)	0.48	0.77	0.35	0.54
LA (25)	0.75	0.56	0.59	0.18
LA (10)	0.68	0.78	0.95	0.77

The effects of different concentration of the drugs on the pharyngeal pumping rates of *C. elegans*. All assays were performed at 23 °C. The worms were treated with varying concentrations of EGCG and LA (10–100 μM) from day one young larvae (L1) until the completion of the assay. Pharyngeal pumps recorded at adult day 2, 4, 6 and 8 are presented to show functional decline of the pharynx muscle ($n=12$ animals for each group). At least three different assays were performed. *$p<0.05$, **$p<0.01$.

noticeable delay in the decrease of sinusoidal (S) movement of these worms as they aged, compared with the non-treated control worms. This effect was not observed during early adulthood, but around day 16, the "aged" worms' behavior seemed to mimic characteristics of worms half their age.

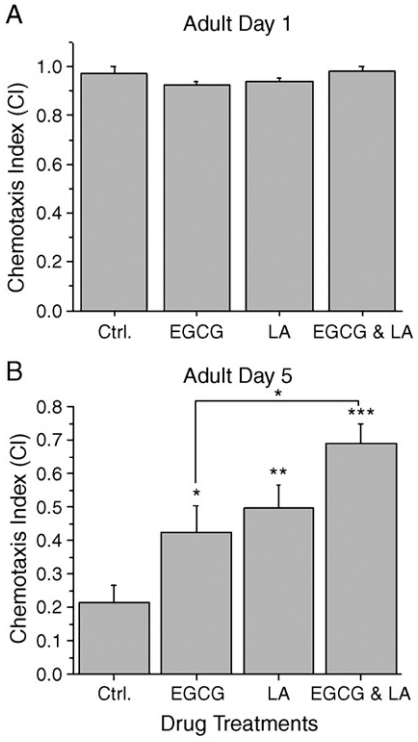

Fig. 4. Effects of EGCG, LA, or their combination, on chemotaxis index (CI) in adult day 1 (A) or adult day 5 (B) *C. elegans*. Age synchronized *C. elegans* were treated from L1 with either 25 μM EGCG, 24 μM LA or a combination of the two antioxidants. For the chemotaxis assays, 1 μl attractive odorant diacetyl were spotted near one edge of the plate plus 1 μl of sodium azide (1 M) to paralyze the worms which arrived at the odorant. After 1 h, worms that reached the odorant were quantitated. All chemotaxis assays were repeated at least three times with independent populations ($n=75$ worms/ treatment with 3 independent trials; LA $p=0.008$, EGCG $p=0.05$). The Chemotaxis Index (CI) was defined as (the number of worms at the attractant − the number of worms at the control spot)/the total number of worms. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Locomotion assays were performed to determine this effect. In this assay, three classes of locomotion phenotypes were scored to track movement (Herndon et al., 2002). Fig. 2C illustrates the three types of movement (A, B and C see Methods) in wild type worms fed with EGCG or LA. The dashed bars represent B movement, which dominates during middle age (day 12) and declines during aging (day 16). The decline of this movement was significantly delayed in worms fed with EGCG at 16 days, compared with control or LA fed worms ($p=0.04$ and 0.01 respectively) (Fig. 2C dashed bars).

3.3. EGCG attenuates decline of pharyngeal pumping rates in *C. elegans*

Pharyngeal pumping is the muscle movement for *C. elegans* food intake and normally declines with age. To investigate if EGCG and LA affect this physiological behavior, pharyngeal pumping was scored in *C. elegans* fed with either EGCG or LA. Animals treated with EGCG continued to exhibit rapid pharyngeal pumping at adult day 4 and older significantly faster than the control group (Fig. 3A), and the group fed with LA, at the same ages (Fig. 3B). Higher concentrations of EGCG (50 μM and 100 μM) further attenuated the rate of decline of pharyngeal pumping in adult day 6 and 8 *C. elegans* (Fig. 3A). Worms treated with 50 μM EGCG showed statistically

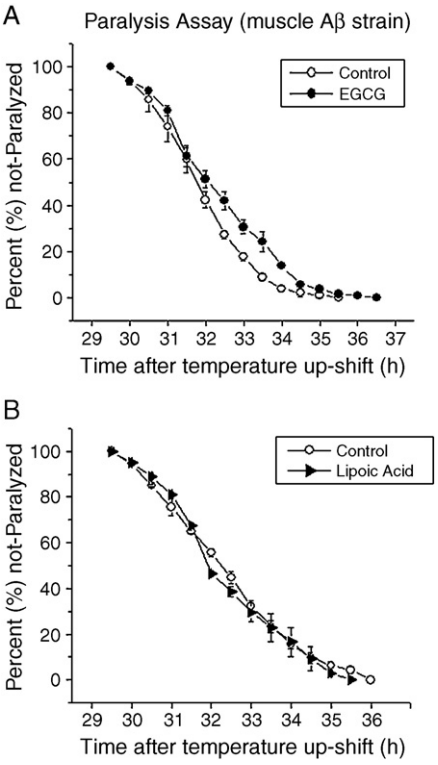

Fig. 5. Paralysis assay in a transgenic *C. elegans* (CL4176) expressing Aβ. Age synchronized CL4176 worms were treated from the egg until the L3 larval stage with 25 μM EGCG (A) or 24 μM LA (B). The animals were allowed to cultivate at 16 °C for 36 h. At the 36 h time point, the temperature was up-shifted to 23 °C. Worms began to paralyze 29 h after the temperature up-shifted and were scored at 30 min interval. Data are expressed as percent (%) worms, which are not paralyzed. EGCG moderately alleviates β amyloid-induced toxicity in mutant *C. elegans* strain.

significant affects on pumping rates at adult day 6 and day 8 compared with the age-matched, untreated controls (50 μM, day 6 and day 8, $p=0.004$ and $p=0.03$ respectively). The worms treated with 100 μM EGCG also show significant effects (see Table 2). Treating the worms with 10–100 μM of LA did not have statistically significant effects on pharyngeal pumping (for details see Table 2).

3.4. LA and EGCG enhance chemotaxis index (CI) in older *C. elegans*

The chemotaxis index (CI) is a measure of the fraction of worms able to transform specific sensory stimuli into goal-directed motor responses (Bargmann et al., 1993). As the worms advance in age, this behavior declines. To investigate the effects of the two antioxidants on age-related changes in chemotaxis, we applied the chemical diacetyl (2, 3 butanedione) as an attractant along with sodium azide, a chemical that paralyzes the worms on contact. The CI was scored at adult day 1 and day 5. As shown in Fig. 4B, both EGCG and LA display a significant enhancement on CI in worms at adult day 5 compared with the untreated controls (EGCG, $p=0.05$; LA, $p=0.008$). Also, combined treatment with LA and EGCG produced a remarkable enhancement of the chemotaxic behavior compared with untreated control worms (total 75 worms per treatment in three independent assays $p=0.00009$) and showed an additive effect when compared with worms fed with EGCG alone (Fig. 4B, EGCG+LA, $p=0.02$). However, there were no discernable effects of either chemical on younger animals tested, at adult day 1 (Fig. 4A).

3.5. EGCG moderately, delays paralysis in Aβ expressing transgenic *C. elegans*

The first group of experiments focused on how the antioxidants alleviate ROS-associated stress during normal aging. The next aim of this study was to determine if EGCG and LA would specifically affect age-associated pathological behaviors. It has been previously demonstrated that EGCG can protect and rescue PC12 cells against Aβ toxicity in a dose-dependent manner (Bastianetto et al., 2006). To determine if EGCG and/or LA could assuage pathological behaviors associated with Aβ accumulation a transgenic *C. elegans* model of AD was used. The transgenic worms display muscle paralysis over time when an inserted transgene is expressed by temperature up-shift from 16 °C to 23 °C, which is a non-permissive temperature for this strain. The time course of the paralysis assay shows that Aβ-induced muscle paralysis was not affected in the worms treated with EGCG in the early hours after paralysis induction (Fig. 5A). However, towards the mid-range and latter half, EGCG did delay paralysis. Although not statistically significant, it is in agreement with the *in vitro* study showing that EGCG attenuates Aβ-induced toxicity in cultured hippocampal neurons (Choi et al., 2001; Levites et al., 2003) and our previous *in vitro* assay that EGCG inhibited Aβ aggregation in solution (data not shown). LA, on the other hand, did not delay Aβ-induced muscle paralysis (Fig. 5B).

4. Discussion

The present study sought to elucidate how two popular antioxidants, EGCG and α-lipoic acid (LA) protect against age-associated behavioral declines in the nematode *C. elegans.* It was found that although EGCG and LA had similar potencies in their antioxidative abilities in the worms (Fig. 1C); EGCG was able to attenuate the rate of age-related pharyngeal contraction decline (Fig. 3A) and moderately alleviated Aβ-induced behavioral pathologies (Fig. 5A). LA, on the other hand, enhanced life span (Fig. 2A) in *C. elegans.* Both antioxidants were able to sustain an increased chemotaxis behavior over the control animals with aging and this increased CI effect is additive (Fig. 4B). Extension of life span in *C. elegans* by LA (Fig. 2A) is supported by a previous report that LA extended life span in the fruit fly *D. melanogaster* (Bauer et al., 2004). The moderate life span extension in wild type *C. elegans* by LA was not observed in *daf-16 (mgDf50)*. The lack of life span extension for this particular mutant suggests that life span extension in the wild type by LA may be dependent on the insulin/IGF-1 signaling pathway in the worms. The insulin/IGF-1 signaling pathway is a major pathway in the nematode that has been found to control reproduction, growth and life span (Wolkow, 2002). The loss of function *daf-16* mutation reduces the worm's life span and suppresses the life span extensions caused by mutations in upstream signaling pathway genes such as daf-2 (Kenyon et al., 1993). Modulation of insulin sensitivity by LA in mammals has been well documented in several studies (Jacob et al., 1999; Evans and Goldfine, 2000) and it is possible that LA increases life span in *C. elegans* via modulating insulin signaling.

Surprisingly, worms that were fed EGCG did not have a significant increase in either the mean or the maximum life span. This may be explained by the notion that administration of EGCG increased fat oxidation in rats (Klaus et al., 2005) and energy expenditure in humans (Dulloo et al., 2000). It has also been demonstrated that several long-lived *C. elegans* mutants increase their life span by decreasing their metabolism (Van Voorhies and Ward, 1999), which is in agreement with finding that higher metabolic rates may increase ROS production, which shortens the life span due to increased oxidative stress. Thus the combined and contradictory actions of EGCG increasing energy expenditure and, at the same time, potently scavenging free radials, results in the worms having a normal life span.

Despite the inability of EGCG to extend life span at concentrations similar to LA, we found that EGCG exhibits the ability to slow the decline in pumping rates in these worms, in a concentration-dependent manor. Inhibition of bacterial growth (Zhang and Rock, 2004) by EGCG may also be a contributing factor to the higher pharyngeal pumping rates shown in worms treated with EGCG. Previous work has shown that *C. elegans* exposed to a food source that was growth inhibited either by UV-irradiation or antibiotics, had higher pumping rates than animals assayed on growing bacterial lawns (Garigan et al., 2002). In comparison, LA has not been shown to have these effects. Although it has been reported that the declines of

pharyngeal pumping and body movement are positively correlated with each other and with life span (Huang et al., 2004), the effects of EGCG did not follow this correlation, probably due to its inherent complexity as both a pro-and anti-oxidant (Azam et al., 2004).

EGCG and LA are both well-known antioxidants, but they apparently modulate age-dependent behaviors differently in *C. elegans*. The additive effects of LA and EGCG on increased chemotaxis in adult day 5 worms support this view. EGCG is a polyphenolic compound with potent antioxidative properties *in vitro* and is also known to have biphasic action, e.g. as pro-oxidant or anti-oxidant depending on the concentration and cellular environment (Mandel et al., 2004; Raza and John, 2005). It has previously been reported that at high concentrations, EGCG induces cytotoxicity and apoptosis in many types of tumor cells and promotes the production of elevated H_2O_2 levels to trigger a distinct apoptotic pathway (Yamamoto et al., 2004). Not only are these effects concentration dependant, but also they are cell-type dependant, differentiating between normal and tumor cells (Yamamoto et al., 2004). However, at lower concentrations (<10 μM), EGCG has been found to protect cells against the detrimental effects of oxidative stress (Bastianetto et al., 2006).

LA, a naturally occurring coenzyme necessary for carbohydrate utilization in ATP production can also be obtained through the diet. In the mitochondria, since LA is reduced to dihyrdrolipoic acid (DHLA) (Wolz and Krieglstein, 1997), DHLA, being a potent extracellular antioxidant, could have contributed to the observed effects reported here. LA has been shown to effectively reverse mitochondrial decay, which is important in the process of aging and cognitive dysfunction (Farr et al., 2003), and a treatment option for AD-related dementia, when taken in combination with standard medicinal treatments (Hager et al., 2001). Since both EGCG and LA are unstable chemicals and metabolized, it is also possible that metabolic by-products of EGCG and/or LA could have contributed to the observed effects reported here. The bioavailability of lipoic acid in humans is approximately 30% (Teichert et al., 1998). No published studies are available on the bioavailability of lipoic acid administered to *C. elegans*.

The nematode *C. elegans* has the ability to differentiate between pathogenic and non-pathogenic bacterial food sources, and varying temperatures and modify its behavior to avoid the adverse conditions (Zhang et al., 2005). The increased chemotaxis index (CI) achieved through LA supplementation in this study (Fig. 4B) is consistent with findings that LA also enhances associative learning in *C. elegans* (Murakami and Murakami, 2005) and aged mice (Farr et al., 2003).

Findings in this study that alleviation of Aβ-induced muscle paralysis by EGCG are in corroboration with previous findings demonstrating that EGCG displays neuroprotective abilities after co-exposure to Aβ in cultured hippocampal cells (Bastianetto et al., 2006). However, treatment with LA in the transgenic *C. elegans* lacked the ability to inhibit Aβ-induced paralysis, although previous research found that LA was able to inhibit Aβ fibril formation and destabilize preformed fAβ from Aβ protein *in vitro* in a dose-dependant manor (Ono et al., 2006). It is possible that specific inhibition of soluble Aβ oligomeric species, rather than Aβ fibril, is the key to delay Aβ-induced paralysis (Wu et al., in press).

In summary, two different types of antioxidants and how they affect age-associated behavioral declines in the nematode *C. elegans* were compared. Their differential effects imply that mechanisms in addition to their status as natural antioxidants may provide benefits for specific diseases/disorders.

Acknowledgements

This study was supported by NIH grant R01AT001928-04 from NIH National Center for Complimentary and Alternative Medicine (YL), and by the Medical Research Institute, San Francisco. The authors would like to thank Dr. Julie Smith and Yanjue Wu for technical assistance and for comments on the manuscript. Also, we would like to thank Drs. Stephane Bastianetto and Remi Quirion of the Douglas Hospital Research Center, McGill University (Quebec, Canada) for initial discussion of the project.

References

Azam S, Hadi N, Khan NU, Hadi SM. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. Toxicol In Vitro 2004;18(5):555–61.

Bargmann CI, Hartwieg E, Horvitz HR. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell 1993;74(3):515–27.

Bastianetto S, Yao ZX, Papadopoulos V, Quirion R. Neuroprotective effects of green and black teas and their catechin gallate esters against beta-amyloid-induced toxicity. Eur J Neurosci 2006;23(1):55–64.

Bauer JH, Goupil S, Garber GB, Helfand SL. An accelerated assay for the identification of lifespan-extending interventions in *Drosophila melanogaster*. Proc Natl Acad Sci U S A 2004;101(35):12980–5.

Biewenga GP, Haenen GR, Bast A. The pharmacology of the antioxidant lipoic acid. Gen Pharmacol 1997;29(3):315–31.

Brenner S. The genetics of *Caenorhabditis elegans*. Genetics 1974;77(1):71–94.

Choi YT, Jung CH, Lee SR, Bae JH, Baek WK, Suh MH, et al. The green tea polyphenol (−)-epigallocatechin gallate attenuates beta-amyloid-induced neurotoxicity in cultured hippocampal neurons. Life Sci 2001;70(5):603–14.

Dulloo AG, Seydoux J, Girardier L, Chantre P, Vandermander J. Green tea and thermogenesis: interactions between catechin-polyphenols, caffeine and sympathetic activity. Int J Obes Relat Metab Disord 2000;24(2):252–8.

Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, et al. Trends in alternative medicine use in the United States, 1990–1997: results of a follow-up national survey. JAMA 1998;280(18):1569–75.

Evans JL, Goldfine ID. Alpha-lipoic acid: a multifunctional antioxidant that improves insulin sensitivity in patients with type 2 diabetes. Diabetes Technol Ther 2000;2(3):401–13.

Farr SA, Poon HF, Dogrukol-Ak D, Drake J, Banks WA, Eyerman E, et al. The antioxidants alpha-lipoic acid and *N*-acetylcysteine reverse memory impairment and brain oxidative stress in aged SAMP8 mice. J Neurochem 2003;84(5):1173–83.

Garigan D, Hsu AL, Fraser AG, Kamath RS, Ahringer J, Kenyon C. Genetic analysis of tissue aging in *Caenorhabditis elegans*: a role for heat-shock factor and bacterial proliferation. Genetics 2002;161(3):1101–12.

Guo Q, Zhao B, Li M, Shen S, Xin W. Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes. Biochim Biophys Acta 1996;1304(3):210–22.

Gutierrez-Zepeda A, Santell R, Wu Z, Brown M, Wu Y, Khan I, et al. Soy isoflavone glycitein protects against beta amyloid-induced toxicity and oxidative stress in transgenic *Caenorhabditis elegans*. BMC Neurosci 2005;6:54.

Hager K, Marahrens A, Kenklies M, Riederer P, Munch G. Alpha-lipoic acid as a new treatment option for Azheimer type dementia. Arch Gerontol Geriatr 2001;32(3):275–82.

Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956;11(3):298–300.

Harman D. The aging process. Proc Natl Acad Sci U S A 1981;78(11):7124–8.

Hekimi S, Guarente L. Genetics and the specificity of the aging process. Science 2003;299(5611):1351–4.

Herndon LA, Schmeissner PJ, Dudaronek JM, Brown PA, Listner KM, Sakano Y, et al. Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. Nature 2002;419(6909):808–14.

Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit Rev Food Sci Nutr 2003;43(1):89–143.

Huang C, Xiong C, Kornfeld K. Measurements of age-related changes of physiological processes that predict lifespan of *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 2004;101(21):8084–9.

Jacob S, Ruus P, Hermann R, Tritschler HJ, Maerker E, Renn W, et al. Oral administration of RAC-alpha-lipoic acid modulates insulin sensitivity in patients with type-2 diabetes mellitus: a placebo-controlled pilot trial. Free Radic Biol Med 1999;27(3–4):309–14.

Jin CF, Shen Sr SR, Zhao BL. Different effects of five catechins on 6-hydroxydopamine-induced apoptosis in PC12 cells. J Agric Food Chem 2001;49(12):6033–8.

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A *C. elegans* mutant that lives twice as long as wild type. Nature 1993;366(6454):461–4.

Klaus S, Pultz S, Thone-Reineke C, Wolfram S. Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. Int J Obes (Lond) 2005;29(6):615–23.

Levites Y, Amit T, Mandel S, Youdim MB. Neuroprotection and neurorescue against Abeta toxicity and PKC-dependent release of nonamyloidogenic soluble precursor protein by green tea polyphenol (−)-epigallocatechin-3-gallate. FASEB J 2003;17(8):952–4.

Link CD, Taft A, Kapulkin V, Duke K, Kim S, Fei Q, et al. Gene expression analysis in a transgenic *Caenorhabditis elegans* Alzheimer's disease model. Neurobiol Aging 2003;24(3):397–413.

Luo Y. Long-lived worms and aging. Redox Rep 2004;9(2):65–9.

Mandel S, Weinreb O, Amit T, Youdim MB. Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (−)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. J Neurochem 2004;88(6):1555–69.

Murakami S, Murakami H. The effects of aging and oxidative stress on learning behavior in *C. elegans*. Neurobiol Aging 2005;26(6):899–905.

Muto S, Yokoi T, Gondo Y, Katsuki M, Shioyama Y, Fujita K, et al. Inhibition of benzo[a]pyrene-induced mutagenesis by (−)-epigallocatechin gallate in the lung of rpsL transgenic mice. Carcinogenesis 1999;20(3):421–4.

Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, et al. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature 1997;389(6654):994–9.

Ono K, Hirohata M, Yamada M. Alpha-lipoic acid exhibits anti-amyloidogenicity for beta-amyloid fibrils in vitro. Biochem Biophys Res Commun 2006;341(4):1046–52.

Packer L, Witt EH, Tritschler HJ. Alpha-lipoic acid as a biological antioxidant. Free Radic Biol Med 1995;19(2):227–50.

Raza H, John A. Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. Toxicol Appl Pharmacol 2005;207(3):212–20.

Smith JV, Luo Y. Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by *Ginkgo biloba* extract EGb 761. J Alzheimer's Dis 2003;5(4):287–300.

Teichert J, Kern J, Tritschler HJ, Ulrich H, Preiss R. Investigations on the pharmacokinetics of alpha-lipoic acid in healthy volunteers. Int J Clin Pharmacol Ther 1998;36(12):625–8.

Van Voorhies WA, Ward S. Genetic and environmental conditions that increase longevity in *Caenorhabditis elegans* decrease metabolic rate. Proc Natl Acad Sci U S A 1999;96(20):11399–403.

Wilson MA, Shukitt-Hale B, Kalt W, Ingram DK, Joseph JA, Wolkow CA. Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. Aging Cell 2006;5(1):59–68.

Wolkow CA. Life span: getting the signal from the nervous system. Trends Neurosci 2002;25(4):212–6.

Wolz P, Krieglstein J. Neuroprotective activity of lipoic and dihydrolipoic acid. In: Fuchs J, Packer L, Zimmer G, editors. Lipoic acid in health and disease. New York: Marcel Dekker; 1997. p. 205–25.

Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 2004;430(7000):686–9.

Wu Z, Smith JV, Paramasivam V, Butko P, Khan I, Cypser JR, et al. *Ginkgo biloba* extract EGb 761 increases stress resistance and extend life span of *Caenorhabditis elegans*. Cell Mol Biol (Noisy-le-grand) 2002;48 (6):725–31.

Wu Y, Wu ZX, Butko P, Christen Y, Lambert MP, Klein WL, et al. Amyloid beta-induced pathological behaviors are suppressed by *Ginkgo biloba* extract EGb 761 and ginkgolides in transgenic *Caenorhabditis elegans.* J Neurosci in press.

Yamamoto T, Lewis J, Wataha J, Dickinson D, Singh B, Bollag WB, et al. Roles of catalase and hydrogen peroxide in green tea polyphenol-induced chemopreventive effects. J Pharmacol Exp Ther 2004;308(1):317–23.

Yanase S, Yasuda K, Ishii N. Adaptive responses to oxidative damage in three mutants of *Caenorhabditis elegans* (age-1, mev-1 and daf-16) that affect life span. Mech Ageing Dev 2002;123:1579–87.

Zhang YM, Rock CO. Evaluation of epigallocatechin gallate and related plant polyphenols as inhibitors of the FabG and FabI reductases of bacterial type II fatty-acid synthase. J Biol Chem 2004;279:30994–1001.

Zhang Y, Lu H, Bargmann CI. Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. Nature 2005;438(7065):179–84.