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Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by *Ginkgo biloba* extract EGb 761

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Abstract. The role of amyloid β-peptide (Aβ) in the free-radical oxidative-stress model of neurotoxicity in Alzheimer's disease (AD) has received much attention recently. In this study, we have employed both *in vitro* and *in vivo* models displaying endogenous Aβ production to study the effects of Aβ on intracellular free radical levels. We employed a neuroblastoma cell line stably expressing an AD-associated double mutation, which exhibits both increased secretion and intracellular accumulation of Aβ when stimulated, as well as transgenic *Caenorhabditis elegans* constitutively expressing human Aβ. A rise in levels of hydrogen peroxide (H_2O_2) was observed in both *in vitro* and *in vivo* AD-associated transgenic models expressing the Aβ peptide compared with the wild type controls. Treatment of the cells or *C. elegans* with *Ginkgo biloba* extract EGb 761 significantly attenuated the basal as well as the induced levels of H_2O_2-related reactive oxygen species (ROS). Among individual EGb 761 components tested, kaempferol and quercetin provided maximum attenuation in both models. Furthermore, an age-dependent increase in H_2O_2-related ROS was observed in wild type *C. elegans*, which is accelerated in the AD-associated *C. elegans* mutant. These results support the hypothesis of the involvement of Aβ and ROS in association with AD.

Keywords: Reactive oxygen species, *Ginkgo biloba*, Amyloid β, Alzheimer's disease, *Caenorhabditis elegans*

Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; AβPP, amyloid β-protein precursor; BA, butyric acid; H_2DCFDA, DCF 2', 7' dichlorodihydrofluoroscein diacetate; DMSO, dimethyl sulfoxide; EGb 761, standard *Ginkgo biloba* extract; NAC, N-acetylcysteine; NGM, nematode growth medium; NSAIDs, nonsteroidal anti-inflammatory drugs; PS, presenilin; ROS, reactive oxygen species; SOD, superoxide dismutase; VitC, vitamin C, or L-ascorbic acid; VitE, vitamin E; WT, wild type; swe/Δ9, transgenic mutant neuronal cells bearing double Swedish mutation of human AβPP and exon-9 deletion mutant PS1.

1. Introduction

Cumulative oxidative stress and the role played by free radicals in the etiology of Alzheimer's disease (AD) and impairment of brain function has been the focus of many recent studies, as well as the interest of the general public wishing to live longer, healthier lives. Research indicates that cellular insults resulting from free radicals may be a major contributor to the neurotoxicity and pathology of AD [9]. With recent findings suggesting links between AD, deposition of Aβ [54,57] and oxidative stress [69], much attention is being devoted currently to antioxidant research.

In addition to theories postulating the involvement of Aβ production and aggregation in the development, progression, and neurotoxicity of AD [13,54], aging has interestingly been postulated to be the greatest risk factor associated with the development of AD [16,43]. The free-radical theory of aging [26] suggests that cumulative oxidative damage at the cellular and tissue level arises as a consequence of normal aero-

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bic metabolism [8,24,26,61,64]. Damage to proteins, lipids, and nucleic acids increases with age [10,25,48]. The involvement of ROS in limiting the life span of an organism has been reported with various models including *Drosophila* and *C. elegans*. Organisms engineered to over-express human superoxide dismutase (SOD) live significantly longer than their normal counterparts [24,31,44,46,61], providing direct support for the free-radical theory of aging. Moreover, transgenic models with deficient SOD activity display an oxidation-sensitive phenotype, with higher protein carbonylation levels [25] and shorter life spans than corresponding wild types [55].

The antioxidant status, or balance of both oxidant and antioxidant functioning of an organism, is important not only for its survival, but also affects the spectrum of damage incurred by oxidation [49]. Success in attenuating free radical production, increased clearance of oxygen radicals, and minimizing resulting damages has been demonstrated through the use of antioxidant therapies [11,12]. As a biological antioxidant, vitamin E prevents the oxidation of polyunsaturated fatty acids and proteins and is an important protective factor in the development of diseases and morbidity related to oxidative processes [42, 47]. In addition to functioning as a powerful antioxidant, studies implicate vitamin E in extension of lifespan in *C. elegans* [1]. Unfortunately, since lipid-soluble vitamins are not easily excreted, excessive intake of vitamin E has been associated with potentially harmful side effects [50]. Recent advances in AD treatment involve the investigation of nonsteroidal anti-inflammatory drugs (NSAIDs) in reducing prevalence of AD among users [2, 32, 62]. However, chronic use of these preparations has been shown to cause such side effects as gastrointestinal toxicity [62]. Given the occurrence of side effects with long-term use, increased interest has turned towards the use of alternative remedies. One such compound, able to cross the blood brain barrier without the side effects of NSAIDs and VitE, is a standardized extract of *Ginkgo biloba* (EGb 761) [20]. Thus far, no mutagenic, carcinogenic, teratogenic, or embryogenic side effects have been demonstrated for EGb 761. Oral LD50 in mice represents 2,100 times the recommended daily dose [73].

Ginkgo biloba, representing the only surviving species of the order Ginkgoales (of the class Gymnospermae) that has existed since the time of the dinosaurs, is one of the oldest thriving trees on the planet [21]. The first written account of the internal use of the leaves of *Ginkgo biloba* for medical purposes dates back to 1505 A.D. [20]. EGb 761 is currently used extensively in clinical trials [18], and is popular in the United States as a natural dietary supplement for memory enhancement. In European countries, the extract is being prescribed for the treatment of peripheral and cerebral insufficiency. The extract inhibits coagulation [58], facilitates attenuates occurring age-related deterioration of cognitive functions in rats [65], and improves degenerative dementias of the Alzheimer's and multi-infarct type [34,35].

The standardized extract EGb 761 consists of 24% flavonols (kaempferol, quercetin and isorhamnetin), and 6% terpene lactones: ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide M, ginkgolide J, and bilobalide B (GA, GB, GC, GJ, and BB, respectively) [27]. Analysis of the individual components by high performance liquid chromatography has been previously documented [23,28], along with quantitative structure-activity relationship analysis of the flavonoids and other phenolic compounds [36] for the hierarchy of radical scavenging abilities. GB has been shown to function as a platelet activating factor antagonist [58] capable of attenuating ROS generation, and therefore contributes to increased circulatory efficiency, both peripheral and cerebral, and decreased incidence of thromboses in individuals [21]. BB has been shown in previous studies to reduce ROS-induced cellular changes, therefore exhibiting antioxidant properties [72]. Existing pharmacological data and clinical trials show EGb 761 to be a potent antioxidant agent and free radical scavenger with neuroprotective effects [18,21,39,52].

We have previously provided evidence of the ability of $A\beta_{(1-42)}$ to aggregate in a cell free system, as well as in $A\beta$-expressing cells, which were both attenuated by EGb 761 [40]. Formation of free radicals by the full length, neurotoxic peptide $A\beta_{(1-42)}$ in solution has been documented [70]. Yatin, et al., reported an increase in ROS production and neurotoxicity by $A\beta$ measured by protein carbonyl levels in cultured embryonic rat hippocampal cells and transgenic *C. elegans*, and the attenuation of $A\beta_{(1-42)}$ -induced ROS and neurotoxicity in neuronal cultures by vitamin E [6,71], a well-known free radical scavenger and antioxidant [36]. A particular advantage of EGb 761 over the well-known, and widely used antioxidant vitamin E is the directionality of its known effects. In contrast to unidirectional antioxidant activity, the biphasic effects of EGb 761 are known to be regulatory and adaptive [18]. The polyvalent activities of EGb 761 give it the advantage of homeostasis sensitivity such as the ability to either dilate or contract blood vessels, or induce the directionality of neurochemical or neuroen-

docrine indicators according to the circumstances [18], a benefit that may not be observed in vitamins or even pharmaceutical preparations targeting a single substrate or receptor.

In this study, we sought to characterize anti-oxidative properties of EGb 761 in both cellular and organism models associated with AD. We examined the antioxidative effect of individual components of EGb 761 as well as in comparison with other known antioxidants. A better understanding of the antioxidative properties of EGb 761 may provide a therapeutic strategy for the treatment of neurodegenerative diseases, as well as those associated with the aging process.

2. Materials and methods

2.1. Reagents

The standardized *Ginkgo biloba* leaf extract EGb 761 commonly used in clinical trials was a gift from Schwabe Pharmaceuticals (Karlsruhe, Germany). The main active components are flavonols (24%) and terpene lactones (6%) [21]. The flavonol glycosides, including kaempherol and quercetin; and the terpene lactones, including GA, GB, GC, GJ, and BB, were obtained from Dr. Ikhlas Khan of the National Center for Natural Products Research (University, MS). Extract constituents were isolated as previously described [23,27]. Stock solutions of VitE and flavonol glycosides were made in 100% ethanol, while stock solutions of EGb 761 and terpene lactones were made in DMSO. Final concentrations of solvents were 0.01% when dissolved in the culture media, or food source of *C. elegans*. $A\beta_{(1-40)}$ was purchased from W.M. Keck Biotechnology (New Haven, CT). Juglone and L-ascorbic acid were purchased from Sigma (St. Louis, MO).

2.2. Cell cultures

The wild type neuroblastoma control cells (N2a), and the N2a cell line stably expressing the double mutations of Swedish mutation AβPP695 and the exon-9 deletion mutant PS1 (swe/Δ9), are a generous gift from Dr. H. Xu of Rockefeller University, NY [67]. The cells were maintained as described previously by Thinakaran et al. [60], in medium containing 50% Dulbecco's Modified Eagle Medium (DMEM) and 50% Reduced Serum Modified Eagle Medium (Opti-MEM), supplemented with 5% fetal bovine serum (FBS), 200 μg/ml of G418, and other antibiotics (Invitrogen, Grand Island, NY).

2.3. Strains of Caenorhabditis elegans worms

Experimental animals were either wild type nematodes (N2) or transgenic mutant nematodes (CL2006 or *mev-1*). The N2 and *mev-1* mutant nematode strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA) and maintained as frozen stock in liquid nitrogen until needed. The transgenic AD-associated nematode strain, a generous gift from Dr. C. Link of University of Colorado, produces $A\beta_{(1-42)}$ constitutively, and has been previously characterized [37]. Worms were grown on solid nematode growth medium (NGM) seeded with a 100 μl spot of *Escherichia coli* (OP50) for food. To prepare age-synchronized animals of either N2, CL2006, or *mev-1*, nematodes were transferred to fresh NGM plates upon reaching reproductive maturity at 3 days of age and allowed to lay eggs for 2 h. Isolated hatchlings from the synchronized eggs (day 1) were cultured on fresh NGM plates under atmospheric conditions in a 20°C temperature-controlled incubator (Sheldon Manufacturing, Model 2005, Cornelius, OR). Adult nematodes were subsequently transferred daily to fresh plates with equivalent food/drug treatments until the cessation of egg laying to avoid confounding of generations. All chemicals for treatment of experimental animals were added directly to the OP50 food source and began when larvae were 2 days old. After 48 h of drug treatment (plus 24 h on juglone when indicated) live animals were harvested by hand picking with sterilized wire loops.

2.4. Analysis of oxidative free radicals

Intracellular ROS were measured in cultured neuronal cells and *C. elegans* nematodes using 2',7'-dichlorodihydrofluoroscein diacetate (H_2DCFDA; Molecular Probes). Non-fluorescent DCF-DA is a freely cell permeable dye, which is readily converted to fluorescent 2'7'-dichlorofluorescein (DCF) due to the interaction with intracellular peroxide (H_2O_2) [51]. At the end of the specified treatment times, cells or worm samples were incubated in the presence of 50 μM H_2DCFDA in phosphate buffered saline (PBS) at 37°C in an FLx800 Microplate Fluorescent Reader (Bio-Tek Instruments, Winookski, VT) for quantification of fluorescence at excitation 485 nm and emission 530 nm. Cell samples were read every 5 min for 50 min, and *C. elegans* samples were read every 10 min for 2 h.

2.5. *Cell culture treatment*

Briefly, cells were cultured in 96-well plates in 200 μl media per well. Cells were either pre-treated or co-treated with 100 μg/ml EGb 761 in culture medium for different treatment times as indicated. Aggregated A$\beta_{(1-40)}$ (1 μM) prepared as described previously [40] was incubated exogenously in culture medium for 24 h. Endogenous expression of Aβ in mutant cells was induced by lowering the FBS concentration in culture media from 5% to 0.5% and adding 1 μM BA for 24 h (Sigma, St. Louis, MO). Oxidative stress was induced in cells by the addition of pro-oxidant juglone (5-hydroxy-1, 4-napthoquinone, Sigma, St. Louis, MO), a quinone that generates superoxide anion ($O_2{}^-$) from molecular oxygen during metabolism [23]. Juglone was added directly to cell culture media at a range of final concentrations between 1 and 40 μM for 15–30 min. Cells were then analyzed for ROS production in a microplate reader.

2.6. *Caenorhabditis elegans treatment*

Age-synchronized *C. elegans* were collected into 100 μl PBS with 1% Tween-20 (PBST) in groups of 30, in eppendorf tubes. The worms were then subjected to equally-timed homogenization (Pellet Pestle Motor, MG Scientific) and sonication (Branson Sonifier 250, VWR Scientific, Suwanee, GA) to break up the outer cuticle, and lyse cellular contents. Immediately prior to reading, 100 μl of H_2DCFDA in PBS was added with a final H_2DCFDA concentration of 50 μM. Samples were vortexed, pipetted into wells of 96-well plates, and analyzed for ROS production in the microplate reader.

2.7. *Statistics*

All data were analyzed by unpaired student t-test using Origin 6.0 software (Microcal Software, Inc., Northampton, MA). Data in this paper are defined as statistically significant if p values are less than 0.05.

3. Results

3.1. *EGb 761 attenuates Aβ-induced ROS increase in wild type neuroblastoma cells*

Studies have shown fibrillated Aβ, typically in the micromolar range, to be neurotoxic and capable of inducing oxidation [5,33,53]. To determine the effect of exogenous Aβ on the levels of H_2O_2, which reportedly mediates Aβ toxicity [7,63], we measured ROS levels in the cells using the DCF assay. Consistent with the results of previous reports in hippocampal cell cultures [4], incubation with exogenous Aβ at a concentration of 1 μM for 24 hrs, significantly increased H_2O_2-related ROS levels by 35% ($p = 0.0002$; $n = 5$, Fig. 1). As a comparison to EGb 761, vitamin C (L-ascorbate), well known for its antioxidant properties, also significantly attenuated Aβ-induced ROS levels. ROS levels in cells exposed to Aβ were attenuated by 19% ($p = 0.0001$), or 51% ($p = 0.00003$) by EGb 761, or L-ascorbate, respectively ($n =$ at least 8).

3.2. *Higher levels of ROS exhibited in both cellular and organismal AD-associated mutant models*

According to the Aβ-induced oxidative stress theory of AD, internal expression of Aβ would cause a rise in the levels of ROS [9]. To determine whether H_2O_2-associated ROS levels are increased by endogenous Aβ expression, we employed both cellular [60] and organism [37,38] models of AD. As expected, Fig. 2(A) shows significantly higher ROS levels in an AD-associated cellular models compared to wild type counterparts. Aβ-secreting mutant cells (swe/Δ9) showed $\sim$3.13-fold higher ROS levels than N2a wild-type cells ($p = 4 \times 10^{-7}$; $n = 8$). Pretreatment with EGb 761 (100 μg/ml) for 48 h significantly attenuated H_2O_2-related ROS levels: in Aβ-secreting mutant cells by 32% ($p = 0.002$) and in wild-type cells by 19% ($p = 0.014$; $n =$ at least 7). We also measured ROS generated in the whole organism using *C. elegans*. The AD-associated mutant worms (CL2006) showed 2.5-fold higher endogenous ROS levels than WT *C. elegans* (N2) ($p = 0.002$; total 240 worms for each strain in 3 independent trials, Fig. 2(B)). Additionally, ROS levels were increased 4 fold in the SOD-deficient mutant nematode strain *mev*-1, compared to the WT controls (data not shown).

3.3. *Pro-oxidant juglone-stimulated increase in ROS levels is attenuated by EGb 761 treatment*

To further characterize the intracellular levels of ROS induced by pro-oxidant juglone, we conducted a time and concentration dependence assay in neuronal cells. In Fig. 3, we show that stimulation of H_2O_2-related ROS in neuronal cells with juglone is both time-, and concentration-dependent. Increased ROS levels are positively correlated both with increasing juglone con-

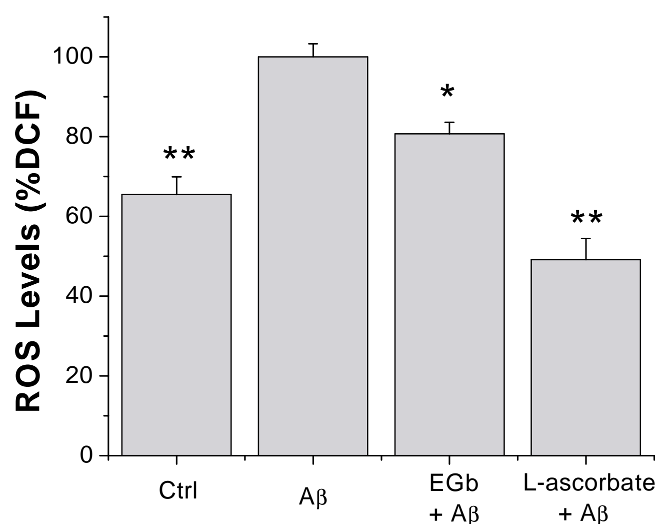

Fig. 1. Effects of exogenous Aβ on ROS levels in neuroblastoma cells. The wild type neuroblastoma cells, grown on 96 well multi-well plates, were left untreated (Ctrl) or exposed to fibrillated amyloid-β (Aβ) at 1 μM for 24 h, or pretreated with either 100 μg/ml *Ginkgo biloba* extract (EGb) or 0.25 mM vitamin C (L-ascorbate) prior to exposure to Aβ. Cells were then analyzed for reactive oxygen species (ROS) production by incubating with 50 μM H_2DFDA for 50 min, followed by measurement of fluorescent DCF production. Results are expressed as percentage of fluorescence (%DCF) relative to cells incubated in the presence of Aβ (Aβ, as 100%, Statistically significance: by independent t-test, *$p < 0.05$; **$p < 0.001$). Results were obtained from 3 independent experiments.

centrations (1–40 μM), and increasing time of juglone incubation (15–45 min) in culture medium. EGb 761 treatment (EGb 761 +) was able to reduce ROS levels in cells stimulated with each concentration of juglone tested: at 40 μM juglone, a 41% attenuation was observed by EGb 761 ($p = 0.006$), at 1 μM, a 22% attenuation by EGb761 ($p = 0.04$). Although the 27% reduction of ROS levels in 10 μM juglone-stimulated cells with EGb 761 was not statistically significant, it did closely approach significance at $p = 0.052$ ($n =$ at least 4).

3.4. *EGb 761 affords cellular protection against rising ROS levels in both pre-treatment or co-treatment conditions*

Through its flavonoid components, scavenging free radicals, and with its ginkgolides helping to counteract the formation of ROS, it is postulated that EGb 761 is capable not only of prevention of oxidative stress through its antioxidant properties, but also of repair of oxidative damages incurred. This hypothesis is strengthened by our recent demonstration of the ability of EGb 761 to prevent serum deprivation-induced DNA fragmentation in neuronal cells in both pre- and post-treatment conditions [56]. Figure 4(A) shows that attenuation of ROS levels in Aβ-secreting cells is achieved with pre- and co-EGb 761 treatment and is concentration dependent. Compared to untreated Aβ-expressing control cells, EGb 761 treatment is shown to be effective both as pre-treatment prior to expression of Aβ, or when administered simultaneous with expression of Aβ. Results also demonstrate that the level of protection against ROS production by EGb 761 treatment follows a distinct concentration-dependent manner. As a pre-treatment, 5–50 μg/ml EGb 761 showed significant ROS attenuation compared to untreated controls. When administered during 24 h expression of Aβ without the benefit of previous treatment, 10–100 μg/ml EGb 761 yielded significantly lower ROS levels than controls ($n = 8$ samples for each EGb 761 concentration tested). Although most studies used a longer incubation (e.g., 48 h) of antioxidant compounds, short incubations of EGb 761 exhibit excellent attenuation of ROS levels. Figure 4(B) shows that when cells were treated with progressively shorter incubation periods of 100 μg/ml EGb 761 (48 h, 24 h, 5 h, 4 h, 3 h, 2 h, 1 h, 30 min) in culture medium, ROS attenuation by EGb 761 was most significant with shortest incubation (30 min).

It has been postulated that the polyvalent action of EGb 761 is due to the combined effects of its individual components. To examine the antioxidant properties of each, we performed ROS assays in neuronal cells treated with six of the individual components. Figure 4(C) shows that maximal attenuation of ROS

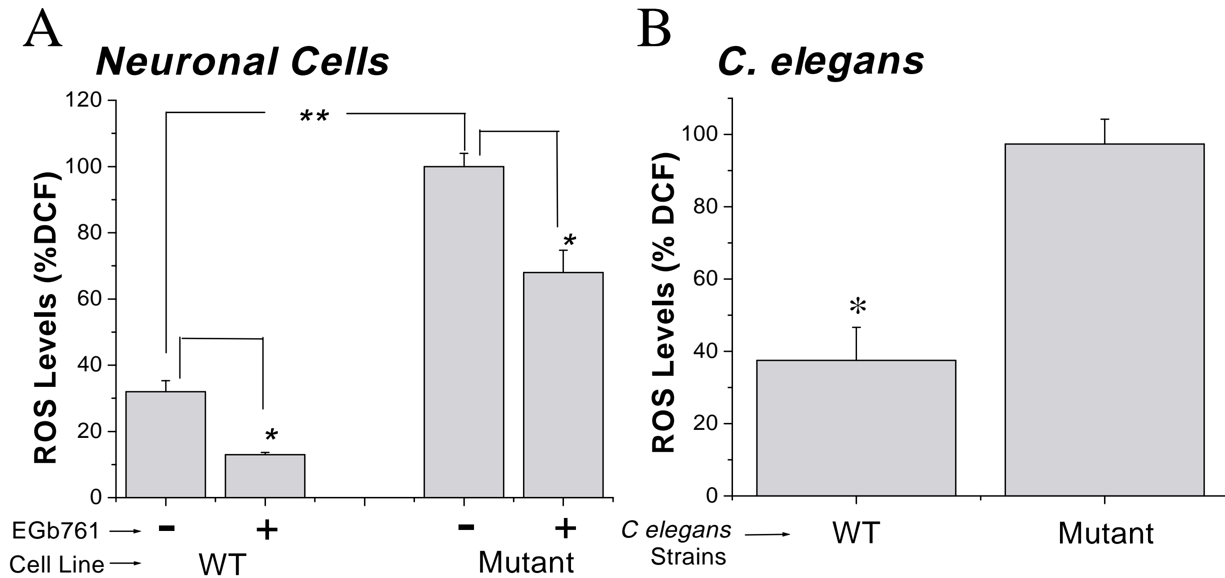

Fig. 2. Comparison of ROS levels *in vitro* and *in vivo*. A. In vitro model: Wild type N2a cells (WT) and the Aβ-secreting mutant cells (Mutant), maintained as described in Methods, were either left untreated (−) or treated (+) with EGb 761 (100 μg/ml) for 48 h. At the end of treatment, the cells were assayed for ROS by the DCF method and results are expressed as percentage of fluorescence (%DCF) relative to untreated mutant cells (as 100%). Results are obtained from 8 independent samples. B. *In vivo* model: Age synchronized groups of wild type *C. elegans* (WT) and AD-associated mutant worms (Mutant), maintained and collected as described in Methods, were assayed at 4 d of age. Results are expressed as percentage of fluorescence (%DCF) relative to mutant worms (as 100%). *Statistically significant (independent t-test, $p < 0.05$; **$p < 0.001$). Results are obtained from 3 independent measurements, representing a total of 480 worms.

in neuronal cells is achieved by treatment with individual flavonoids. Among the six constituents tested, kaempferol (K) and quercetin (Q) show the most impressive attenuation of ROS levels. ROS levels were attenuated by 39% by the whole extract EGb 761 ($p = 1 \times 10^{-6}$). Attenuation by flavonoid components was even more dramatic: 52% attenuation by kaempferol ($p = 2 \times 10^{-8}$) and 44% attenuation by quercetin ($p = 1 \times 10^{-8}$). Treatment with the terpene lactone constituents attenuated ROS levels by 12% with ginkgolide B (GB) ($p = 0.03$) and by 13% with bilobalide (BB) ($p = 0.02$). Although reduction of ROS is statistically significant in the case of GB and BB, the resulting levels are clearly not as dramatic as those achieved by the flavonoid components. To test possible synergistic activities among the constituents, we also treated the cells with combinations of the individual components. Kaempferol and quercetin do not appear to have an additive effect, with a 55% attenuation of ROS compared to untreated control; nor does the combination of bilobalide and kaempferol with a 45% attenuation of ROS compared to untreated control. Rather, each combination appears to be influenced most significantly by the kaempferol, which exhibited a 52% ROS attenuation when tested independently ($n = 2$ independent trials with 8 separate samples of each component tested).

In order to understand the antioxidant properties of EGb 761, response of ROS levels to other known antioxidants was measured after a 30 min incubation of VitC, VitE or N-acetylcysteine (NAC) on neuronal cell cultures. Figure 4(D) shows that in comparison to untreated controls (set as 100%), VitC significantly attenuated ROS levels in neuronal cells in a concentration dependent manner. A 38% attenuation was achieved by 0.25 mM VitC ($p = 3 \times 10^{-5}$), a 42% attenuation by 0.50 mM VitC ($p = 1 \times 10^{-5}$) and a 47% attenuation by 1.0 mM VitC ($p = 3 \times 10^{-6}$). VitE, a lipid-soluble vitamin, is widely prescribed and taken for its antioxidant properties [47,59]. In our study, VitE treatment at 100 μg/ml showed a significant, 45% attenuation of ROS ($p = 5 \times 10^{-6}$) compared to untreated controls (set as 100%) in neuronal cells. NAC, a weaker antioxidant which contributes to the regeneration of glutathione [3] requires much higher doses to achieve the same benefits as some other antioxidant supplements [47]. This was confirmed by our study, in which 200 μM NAC did significantly attenuate ROS levels by 33% ($p = 3.47 \times 10^{-4}$) compared to untreated neuronal cell controls (as 100%), however not

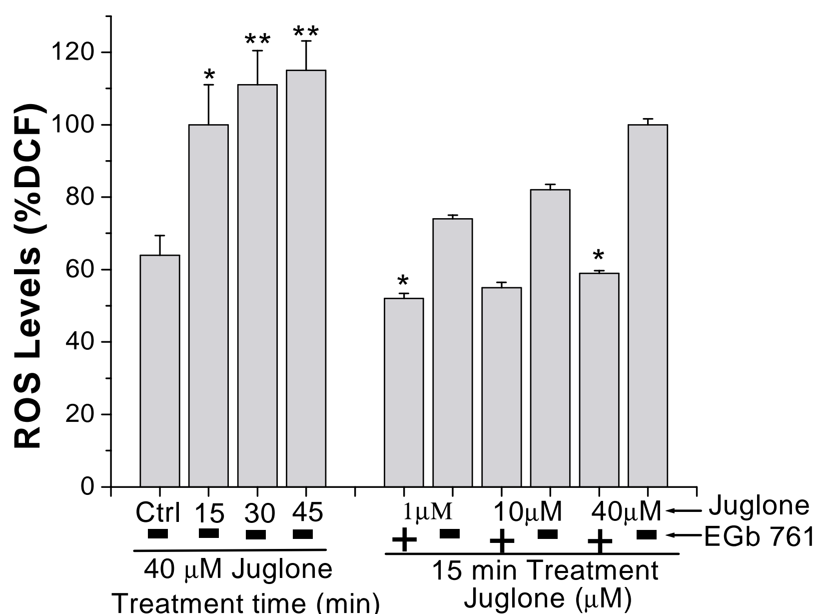

Fig. 3. ROS levels in neuronal cells induced by juglone. ROS production in neuronal cells was stimulated with 1–40 μM pro-oxidant juglone for incubation periods of 15–45 min. As indicated in the graph, results of juglone time dependence are expressed as percentage of fluorescence (%DCF) relative to control (as 100%). Results of juglone concentration dependence are expressed relative to control (no juglone) samples stimulated with 40 μM juglone without EGb 761 treatment (EGb 761 $-$) (as 100%). ROS levels for samples left untreated (EGb 761 $-$) or pretreated with EGb 761 for 48 h prior to juglone stimulation (EGb 761 $+$) are shown for each juglone concentration tested. *Statistically significant (independent t-test, $p < 0.05$; **$p < 0.001$). Results are obtained from at least 4 independent samples.

to the extent of vitamins C or E ($n = 8$ samples for each condition tested).

3.5. *Kaempferol and Quercetin offer remarkable protection against increase of ROS levels in vitro and in vivo*

The studies mentioned above have demonstrated an increase in ROS levels by exogenous and endogenous Aβ (Figs 1 and 2(A)), or by pro-oxidant juglone (Fig. 3), in both cellular and organismal models associated with AD (Fig. 2). Further, EGb 761, kaempferol and quercetin exhibited the strongest attenuation of ROS (Fig. 1–4). Next, we compared the protection afforded by EGb 761, kaempferol and quercetin in both models. Figure 5(A) indicates that significant attenuation of ROS was demonstrated with each treatment of neuronal cells. Following pretreatment with 100 μg/ml of either EGb 761, kaempferol or quercetin, cells were either stimulated with 40 μM juglone, known for its ability to facilitate the production of free radicals [29], or with 1 μM BA to stimulate the expression of Aβ. Compared with untreated Aβ-expressing control cells (set as 100%), ROS levels were significantly attenuated in Aβ-expressing cells by 17%, 78% and 65% in cells treated with EGb 761 ($p = 4 \times 10^{-4}$), with kaempferol ($p = 2 \times 10^{-12}$), and with quercetin ($p = 3 \times 10^{-11}$), respectively. Compared with juglone-induced controls without EGb 761 treatment (set as 100%), ROS levels were again significantly attenuated in juglone-induced cells by EGb 761 (61% attenuation, $p = 6.42 \times 10^{-4}$), kaempferol (40% attenuation, $p = 7.5 \times 10^{-3}$) and quercetin (19% attenuation, $p = 0.0084$; $n = 8$ for kaempferol and quercetin; $n = 40$ for EGb 761).

Figure 5(B) demonstrates that although reduction of ROS levels in AD-associated nematodes treated with EGb 761 was not statistically significant by definition, there was a ROS reduction of approximately 21% ($p = 0.18$). The pattern shown in this *in vivo* ROS study of AD-associated *C. elegans* closely resembles that of the *in vitro* assay in Aβ-expressing neuronal cells in Fig. 5(A), with more significant attenuation achieved by the flavonoids than EGb 761. Treatment by isolated flavonoid components kaempferol (Kaemph) or quercetin (Querc), or vitamin C (L-ascorbate) significantly attenuated ROS levels when compared to untreated transgenic control nematodes (Attenuation vs. Ctrl: kaempferol by 69%, $p = 2.81 \times 10^{-3}$; quercetin by 44%, $p = 0.046$; L-ascorbate by 30%; $p = 0.015$; in two independent trials with at least 60 worms/group).

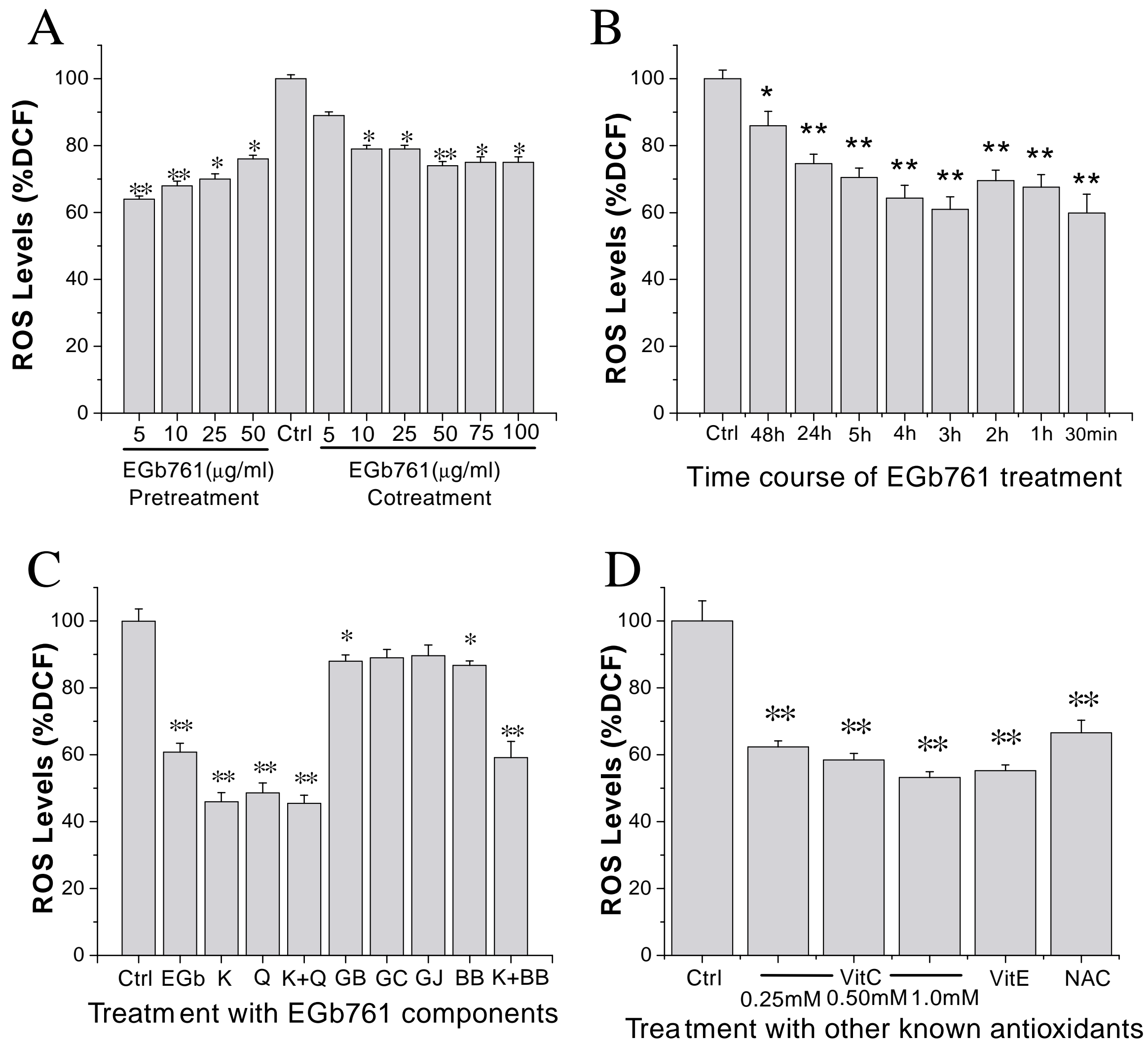

Fig. 4. Comparison of ROS levels in neuronal cells treated with EGb 761 at variable times, concentrations, and combinations of constituents. Except for Fig. 4(A), ROS levels were measured in mutant cells under non-BA stimulated, standard cell culture conditions as described in Methods, and expressed as percentage of fluorescence relative to untreated control cells (Ctrl) (as 100%). A. Aβ-secreting cells (Ctrl) were either pretreated for 48h with EGb 761 (5–50 μg/ml, as indicated in the graph) prior to stimulation with 1 μM BA to express the transgene, or given EGb 761 (5–100 μg/ml, as indicated in the graph) during stimulation with 1 μM BA without pretreatment. Results are expressed as percentage of fluorescence (%DCF) relative to untreated Aβ-secreting cells (Ctrl, as 100%). B. The neuronal cells were treated with EGb 761 (100 μg/ml) in culture medium over a range of time periods (30 min–48 h, as indicated in the graph) followed by measurement of ROS levels. Data are expressed as % DCF of ROS relative to untreated controls (Ctrl, as 100%).C. The neuronal cells were treated with individually isolated or combined constituent fractions of EGb 761 (final concentration 100 μg/ml each) for 48 h followed by measurement of ROS levels. Flavonols: kaempferol (K), quercetin (Q); Terpene lactones: GB, GC, GJ, or BB. Untreated controls (Ctrl) are arbitrarily set to 100%. D. The neuronal cells were treated for 30 min with other known antioxidants, then measured for ROS levels: VitC (0.25 mM, 0.50 mM, 1.0 mM), VitE (100 μg/ml), and NAC (200 μM). *Statistically significant (independent t-test, $p < 0.05$; **$p < 0.001$). Results were obtained from at least 8 independent samples for each compound tested.

3.6. *The levels of ROS increase with age in wild type worms, with a faster rate in AD-associated C. elegans mutants*

It has been well accepted that oxidative stress is a feature of cellular aging [48], and intracellular free radical levels increase with age in mitochondria [43]. To test the hypothesis that ROS levels are accumulated at a higher rate in Alzheimer's models, we compared ROS levels in both wild type and AD-associated *C. elegans* nematodes. Results of Fig. 6 indicate that ROS produc-

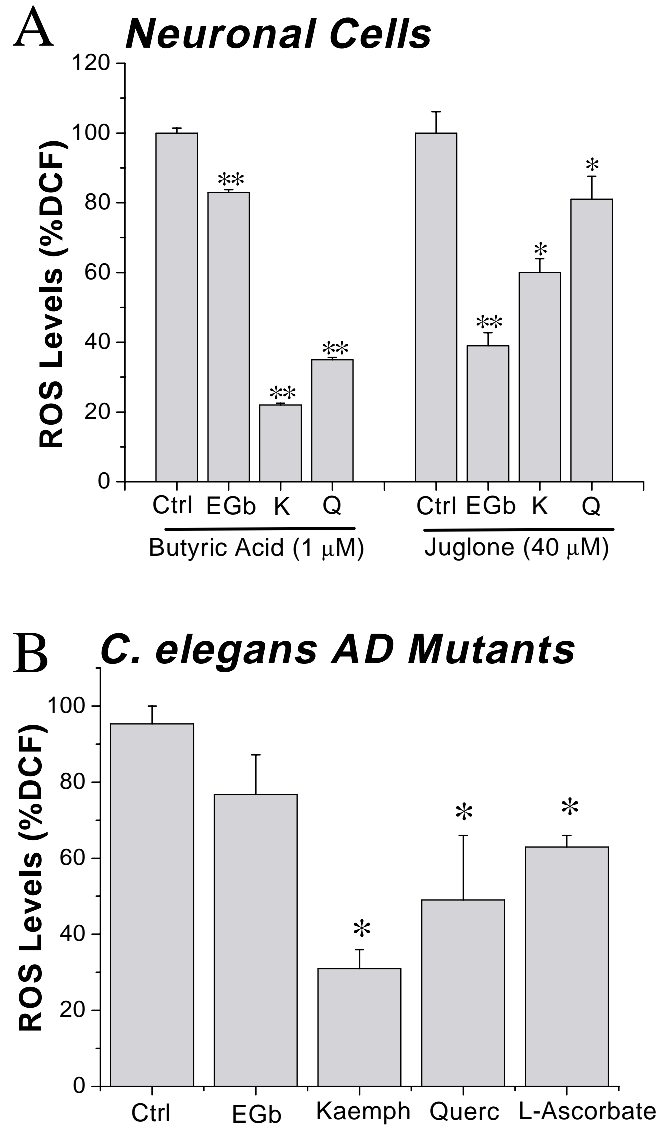

Fig. 5. Flavonoid components of EGb 761 provide maximal attenuation of ROS to *in vitro* and *in vivo* AD models. A. *In vitro*: Transgenic neuronal cells, maintained as described in Methods, were treated for 48 h. Cells were treated with 100 μg/ml of either EGb 761 (EGb), kaempferol (K), or quercetin (Q), then either stimulated for transgene Aβ-expression by the addition of 1 μM BA for 24 h, or stimulated with 40 μM of pro-oxidant juglone for 15 min. Cells were assayed for ROS with results expressed as percentage of fluorescence (%DCF) relative to untreated controls (Ctrl) at 100%. Results are obtained from at least 8 independent samples for each test condition. B. *In vivo:* Age synchronized groups of AD-associated *C. elegans* mutants, maintained as described in Methods were left untreated (Ctrl), or treated with 100 μg/ml of EGb 761, kaempferol (Kaemph), or quercetin (Querc), or 0.25 mM VitC added directly into nematode food source (OP50) from 2 days of age until 4 days of age. At least 60 animals from each group were analyzed for ROS production, and results are expressed as percentage of fluorescence (%DCF) relative to Ctrl. *Statistically significant (independent t-test, $p < 0.05$; **$p < 0.001$). Results were obtained from 2 independent experiments and represent a total of 480 worms.

tion is positively correlated with age in both strains, and that AD-associated mutant animals accumulate ROS at a more rapid rate than wild type animals, indicated as arrow bars in the graph ($n = 2$ independent trials with 60 worms/group tested, 1,200 worms total).

4. Discussion

4.1. ROS levels in whole organisms

Numerous research groups [9,43,48] have conducted studies on the possible relevance of free radical dam-

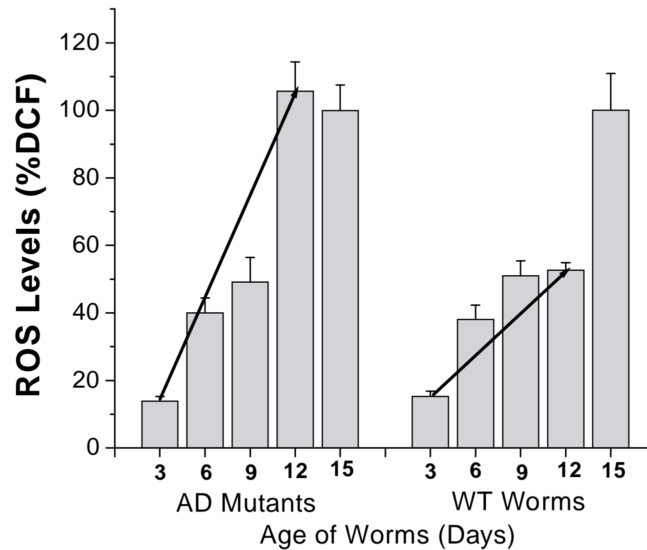

Fig. 6. Age-dependent ROS production is accelerated in AD *C. elegans* model. Both WT and AD-associated mutant nematodes were maintained separately as described in Methods, as age-synchronized groups ranging from 3–15 days old. Experimental groups were homogenized and sonicated, then immediately analyzed for ROS production. Results are expressed as percentage of fluorescence (%DCF) relative to 15 day-old AD mutant animals. Arrows indicate rate of ROS elevation from 3—12 d of age for both nematode strains. Results were obtained from 2 independent experiments and represent a total of 1,180 worms.

age to the development of neurodegenerative diseases. Even though research and discoveries in the area of free radicals is growing exponentially, there appears to be a consensus among researchers that the actions and measurements of free radicals in biological tissues *in vivo* remains a major hurdle [10]. Additionally, direct detection of free radicals is challenging due to the short-lived nature of these highly reactive molecules. Ongoing oxidative damage is thus generally analyzed by measurement of secondary products, including derivatives of amino acids, nucleic acids and lipid peroxidation [14]. To our knowledge, we are the first to directly show the levels of H_2O_2 – related ROS generated within a whole organism in an age-dependent fashion, and with a direct correlation between ROS production in both *in vitro* and *in vivo* models. Results of this study demonstrate the same pattern of ROS attenuation by the *Ginkgo biloba* extract EGb 761 and its flavonoid constituents kaempferol and quercetin both *in vitro* and *in vivo*, with effective and significant attenuation in AD-associated transgenic models. The correlation between results demonstrated *in vitro* in neuronal cells and *in vivo* in an animal model with nematodes, confirms that EGb 761 is effective as an antioxidant and radical scavenging agent within a whole-organism system undergoing normal metabolic processing, capable of modulating responses to external and internal stimuli. Of particular interest is the reduction of H_2O_2 free radical levels by EGb 761 in transgenic AD-associated models expressing the Aβ peptide.

4.2. ROS levels in Aβ-expressing models

Some research groups have investigated the relationship between free radical production and Aβ by exposing neuronal cultures to exogenous synthetic A$\beta_{(1-42)}$ [68]. The present study provides direct evidence of elevated ROS in transgenic neuronal cells secreting endogenous Aβ as an *in vitro* cellular model of AD-associated neurons [60], as well as *in vivo* evidence of elevated ROS in transgenic *C. elegans* expressing constitutive Aβ [37]. Additionally, we used transgenic *C. elegans* nematodes to show that free radical levels not only increase over the lifespan of an organism, but also that the rate of ROS accumulation is accelerated in an AD-associated model compared to wild type animals. We further confirmed the validity of this assay by employing the *mev-1* mutant, in which the SOD activity is half that of the wild type nematodes. The ROS levels in the *mev-1* mutants are approximately four fold that of the wild type controls (data not shown).

Although *in vivo* expression of Aβ is in muscle cells of transgenic CL2006 *C. elegans* mutants, a positive correlation is shown between ROS levels and muscle degeneration presenting as paralysis [22]. Since cellular damage resulting from ROS production is believed

to be one of the early events in the progression of neurodegenerative diseases such as AD [16,24,30,33,48], attenuation of ROS by EGb 761 in both *in vitro* and *in vivo* AD-associated models is of significant importance.

4.3. EGb 761 as an antioxidant

As a major chain-breaking antioxidant [42], VitE is widely used as one of the first lines of defense against lipid peroxidation and free radical damage, and has been the focus of many studies [1,6,12,15,69]. The lipid-soluble antioxidant VitE has the advantage of being able to maneuver through the fatty parts of cellular membranes. However, this simultaneously poses a disadvantage for VitE in limiting its ability to scavenge peroxyl radicals to only hydrophobic domains. In contrast, L-ascorbic acid (VitC), another well-recognized antioxidant [47], is a water-soluble vitamin. VitC has been called the "hub of the antioxidant network" and the "link connecting the fat-soluble antioxidants to the water-soluble antioxidants" due to its ability to recycle the fat-soluble VitE [47].

This research focuses on the use of the *Ginkgo biloba* extract EGb 761 as an antioxidative agent in AD-associated models. For physiological relevance, Maitra et al. evaluated the peroxyl radical scavenging activity of EGb 761 in both aqueous and lipid environments [41]. Results indicate the ability of EGb 761 to gain access to both the aqueous and lipid environments in which oxidative stress can occur [41]. Since EGb 761 is able to effectively scavenge free radical species in both cytosolic (hydrophilic) and membrane (hydrophobic) domains of cells [47], it is plausible to suppose that the polyvalent actions of this extract may afford more effective antioxidant protection than the lipid-soluble vitamin E or water-soluble vitamin C.

Interestingly, regarding the attenuation of H_2O_2 free radicals, the antioxidant capabilities of VitC outperformed that of the whole extract EGb 761 in this study. Compared to cells treated with EGb 761, levels of H_2O_2 – related ROS in VitC-treated cells were 8% lower in neuronal cell cultures (Figs 4C and D), 9% lower in AD-associated *C. elegans* (Fig. 5B) and 32% lower in neuronal cells exposed to exogenous Aβ (Fig. 1). One explanation for this large difference may be due to a direct scavenging of Aβ peptide-generated ROS by VitC in the culture medium therefore potentially reducing the peptide to a less neurotoxic moiety. Additionally, the challenge of transporting a water-soluble vitamin through the hydrophobic cellular membrane could theoretically allow a higher concentration of the vitamin to remain in the media to interact with ROS-generating agents. Attenuation of H_2O_2 free radicals was 6% greater in VitE-treated neuronal cells than EGb 761-treated cells (Figs 4C and D). However, due to the strictly hydrophobic nature of VitE, a similar scenario may be represented, in that the difficulty of satisfactorily maintaining VitE in an emulsion with a ROS-generating agent may be less than optimal. Obviously, this potentially interferes with the ability of VitE to regulate the antioxidant status of the aqueous extracellular environment.

It is interesting to note that increasing concentrations of EGb 761 as pretreatment is negatively correlated with ROS attenuation; whereas when EGb 761 is given concurrently with expression of Aβ, higher EGb 761 dosage yields a positive correlation with ROS attenuation (Fig. 4A); and that cells treated with the shortest incubation period demonstrated the most significant attenuation of ROS (Fig. 4B). These could be due to the effects of EGb 761 scavenging pre-formed free radicals versus preventing the generation of free radicals. The same explanation may apply to another observation (Fig. 5A) that the flavonoids kaempferol and quercetin performed far better than EGb 761 in the cells endogenously expressing the Aβ-peptide, while the opposite was shown in cells externally stimulated to produce ROS by juglone. The presence of other active constituents of the whole extract (EGb 761) has been known to provide additional anti-oxidative properties [19].

4.4. EGb 761 as a multivalent modulator

The ability of EGb 761 to facilitate adaptive responses, rather than unidirectional stimulation or inhibition [18], is certainly one of the most intriguing aspects of EGb 761, and is postulated to be due to the polyvalent or synergistic activities of this multi-constituent extract. *Ginkgo biloba* studies ranging from cellular to behavioral and pharmacokinetic levels, have indicated effectiveness of EGb 761 at almost every level of life [18,20].

Given the ability of EGb 761 to provide antioxidant protection in both hydrophilic and hydrophobic environments, this extract exhibits significant promise as a therapeutic agent capable of antioxidative regulation in a physiologically relevant environment. We supported this hypothesis by providing evidence of whole-organism ROS reduction in an *in vivo* animal model. In addition to the antioxidant proper-

ties of the whole extract EGb 761, much work has been done in an effort to determine the mechanism of action of the individual constituents of the EGb 761 extract [18,19,21,56,58]. Analysis has shown the flavonoid components of the extract to have strong antioxidant properties [17,21,47]. In this study, we provide evidence confirming this in both cell culture and whole-organism models. Not only do the flavonoid components of kaempferol and quercetin outperform the whole extract EGb 761 (Figs 4C, 5A and 5B), they also provide greater attenuation than vitamins C or E (Fig. 5B). Most surprisingly, these flavonoids provide greater free radical protection in models associated with AD mutations (Figs 5A and B) than the wild type counterparts, measured under non-stressed, physiological conditions (Fig. 4C). This is consistent with our previous report of the protection afforded by EGb 761, particularly under stress-induced conditions [66]. As the anti-oxidative activity of EGb 761 appears to be related to kaempferol and quercetin, it is possible that other plants (e.g. onions, berries, tea, etc.) with high contents of these flavonoid substances [45] would be potent anti-oxidants in both models used in this study. Further studies of the specificity of attenuating endogenous ROS by EGb 761 and by other plant flavonols would provide insight into the neuroprotective mechanisms of EGb 761.

In summary, the degree of agreement exhibited by the *in vitro* and *in vivo* models presented in this study increases our level of confidence in this assay as a measure of the steady state levels of ROS; and also strengthens the hypothesis that the production and modulation of free radicals is significantly altered and plays a significant role, in organisms associated with AD. In addition to an often-assumed lack of confidence in, or ineffectiveness of *in vitro* studies to accurately simulate physiological environments, we realistically recognize the challenges of measuring exact levels of the transient and dynamic oxidative free radical molecules. This study provides a significant advance in free radical research by capturing the steady-state level of H_2O_2 – related ROS in a whole-organism model of *C. elegans*, by providing evidence that the levels of ROS increase over the life span of a whole organism, and by showing that the rate of ROS accumulation is increased in the AD-associated animals in comparison to their wild type counterparts (Fig. 6).

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