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Chronic antioxidant therapy reduces oxidative stress in a mouse model of Alzheimer's disease

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Abstract

Oxidative modifications are a hallmark of oxidative imbalance in the brains of individuals with Alzheimer's, Parkinson's and prion diseases and their respective animal models. While the causes of oxidative stress are relatively well-documented, the effects of chronically reducing oxidative stress on cognition, pathology and biochemistry require further clarification. To address this, young and aged control and amyloid-β protein precursor-over-expressing mice were fed a diet with added R-alpha lipoic acid for 10 months to determine the effect of chronic antioxidant administration on the cognition and neuropathology and biochemistry of the brain. Both wild type and transgenic mice treated with R-alpha lipoic acid displayed significant reductions in markers of oxidative modifications. On the other hand, R-alpha lipoic acid had little effect on Y-maze performance throughout the study and did not decrease end-point amyloid-β load. These results suggest that, despite the clear role of oxidative stress in mediating amyloid pathology and cognitive decline in ageing and AβPP-transgenic mice, long-term antioxidant therapy, at levels within tolerable nutritional guidelines and which reduce oxidative modifications, have limited benefit.

Keywords: *Alzheimer's disease, amyloid-β, antioxidant, R-alpha lipoic acid, transgenic mice*

Abbreviations: *AD, Alzheimer's disease; Aβ*, amyloid-β; AβPP, amyloid-β protein precursor; HO-1, heme oxygenase-1; HNE, 4-hydroxynonenal; NFT, neurofibrillary tangles; LA, R-alpha lipoic acid; TBS, Tris-buffered saline

Introduction

Oxidative modifications have been proposed as one biochemical change that could lead to the neuropathology and neuronal dysfunction and death found in Alzheimer's disease (AD) [1–3]. Early work focused on late stage oxidative damage, such as advanced glycation end-products [4] and carboxymethyllysine [5] as the first oxidative modifications found in neurofibrillary tangles (NFT) and plaques containing tau and amyloid-β fibrils (Aβ), respectively. Later studies showed tau phosphorylation, notably the Alz-50 epitope often regarded as an 'early' marker of NFT development [6], occurring coincident with markers of oxidative stress [7,8] and led to the notion that oxidative modification, particularly lipid peroxidation products, such as 4-hydroxynonenal (HNE) and related compounds [8], were involved in the fibrillization and aggregation of tau [9,10]. Consistent with

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this, HNE-protein adducts are present at higher levels within pyramidal neurons of AD as compared to controls [11]. Of note, damage to neuronal nucleic acids, in particular RNA, is significantly increased in AD, with the highest levels seen before the development of large numbers of NFT and amyloid plaques, suggesting oxidative damage as one of the earliest events in disease pathogenesis [12–15].

Of clear importance is the fact that oxidative imbalance is found at all stages of AD [16,17]. Diseased neurons can remain viable for 10 years or longer [18,19] and as such must have sufficient protective mechanisms to maintain normal homeostasis. However, as a neurodegenerative disorder, at some point in the disease, the oxidative insults may overwhelm cellular antioxidant defense systems leading to cellular dysfunction and death. Unfortunately, antioxidant therapy studies to date have focused on either administering antioxidants to patients well into the disease course or for shorter durations and have resulted in clinically equivocal or slight measurable benefit [20–22]. On the other hand, while a comprehensive review of many trials utilizing antioxidants for various disorders has shown that, as a group, there was no adverse effect, certain compounds under specific circumstances were correlated with higher mortality rates [23]. One reason may be that phenolic antioxidants, as well as others, produce pro-oxidant intermediates while scavenging free radicals, which can be counterproductive [24]. We hypothesize that the effects of antioxidant therapy work to help cellular function and survival, but only with selected antioxidants and at levels and timepoints in the disease course that will augment endogenous oxidative responses [25].

The antioxidant R-alpha lipoic acid (LA) has been suggested as a therapeutic that might act to increase the production of acetylcholine or as a chelator of redox-active metals or even to combat the accumulation of lipid peroxidation products [26,27]. In this respect, LA used in conjunction with acetyl carnitine protects neuronal cells *in vitro* from the effects of HNE-toxicity [28] and, at least in some cases, this was shown to be protective via cell signalling mechanisms including the extracellular signal-related kinase pathways, which are dysregulated in AD [29–31]. Importantly, the antioxidant capacity of lipoic acid and its readily reduced form dihydrolipoic acid have been shown to fully attenuate the deleterious phenotype of vitamin E deficiency in a mouse model [32].

In this study, we found that while the long-term administration of LA in control mice and mice overexpressing amyloid-β protein precursor (AβPP) did significantly lower the levels of oxidative markers in both wild type and transgenic mice, there were neither effects on cognition as measured by the Y-maze nor on Aβ load. Overall, the findings support the efficacy of long-term antioxidant supplementation to combat the effects of oxidative modifications, but a functional role of these modifications on normal ageing and diseased states is not supported.

Materials and methods

Animals

Twenty mice (B6SJL) were available for this study, nine wild type and 11 transgenic for AβPP Tg2576 [33], aged 6.25–11.5 months at the onset of the study. Wild-type and transgenic mice were divided into groups receiving either normal or an LA supplemented diet (see Table I). All animals were housed individually in microisolator cages in the same room on a schedule of 12 h light and dark and offered environmental enrichment. Intake of food, water, weight, behaviour and enrichment were all carefully monitored for the 10-month period of study. Body weights were recorded every month and *r*-values obtained. No group (normal or LA-enriched food) showed any significant trend. One animal exhibited very low levels of activity and one animal exhibited continuous running in circles in his cage while all others appeared normal. Every other day the animals were observed by either laboratory personnel or animal centre technicians and veterinarians. All experiments were approved by the institutional animal use and care committee (Case Western Reserve University IACUC).

Diet

Administration of the supplement through the food was chosen to mimic a routine therapy for human

Table I. Transgenic status, diet type, age at onset of experimental diet and age at the end of the study for the mice used.

Mouse type	Diet	Age at onset (months)	Age at end (months)
Wild-type	Normal	6.25	16.25
Wild-type	Normal	6.25	16.25
Wild-type	Normal	7	17
Wild-type	Normal	11.5	21.5
Wild-type	Lipoic acid	7	17
Wild-type	Lipoic acid	7	17
Wild-type	Lipoic acid	8.5	16†
Wild-type	Lipoic acid	11	21
Wild-type	Lipoic acid	11.5	21.5
Tg2576	Normal	6.25	16.25
Tg2576	Normal	6.25	16.25
Tg2576	Normal	7	14.5†
Tg2576	Normal	8.5	18.5
Tg2576	Normal	11.5	21.5
Tg2576	Normal	12	22
Tg2576	Lipoic acid	6.25	16.25*
Tg2576	Lipoic acid	6.25	16.25
Tg2576	Lipoic acid	7	17
Tg2576	Lipoic acid	11.5	21.5
Tg2576	Lipoic acid	11.5	21.5

*mouse not included in behaviour testing due to inactivity. †died before the end of the study.

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application and has been shown to cross the blood–brain barrier [34]. LA (98.97%) was supplied by NeoGen (Lansing, MI) and incorporated into pelleted AIN93M diet (MP Biomedicals, Solon, OH) at a concentration for an expected dietary intake of 30 mg/kg per day. The LD_{50} for rats was found to be $\sim$ 500 mg/kg [35] and studies using between 25–100 mg/kg showed measurable reductions in oxidative stress in other models [26,36]. For this project, all food was administered by the laboratory personnel. Throughout the study, new food was routinely administered to all animals. The weight of fresh food and any food remaining, including any crumbs that could be collected, was recorded. Thus, the approximate amount of food eaten and thus the true daily dosage of LA was determined. At the same time, all animals were weighed to note any physical differences resulting from the two diets. Comparing the beginning and ending weights showed no significant weight changes between any of the groups or diets.

All animals ate very well and no statistically significant differences were seen between the animals on the normal diet vs the LA diet ($p = 0.85$ for the transgenic and $p = 0.35$ for the wild-type mice). It was determined that the animals actually ingested, on average, 4.2 ± 0.7 grams of food per day, slightly less than the proposed 5 g/day.

Toxicity test

Since this project was a long-term study, a toxicity test was designed to test any ill-effects of the LA supplemented diet. Using reported LD_{50} rates of 400–500 mg/kg body weight in rats, two additional mice were fed 5 $\times$ and 15 $\times$ the experimental dosage or 150 mg/kg and 450 mg/kg for 2 weeks. The mice ate the food normally and showed no behavioural or physical ill effects, up to an additional 6 weeks.

Behaviour testing

Y-maze analysis has been shown to be a reliable, non-invasive test to determine cognitive changes in the Tg2576 mouse [37–39]. The Y-maze apparatus consisted of three arms 32 cm (long) $\times$ 10 cm (wide) with 26-cm walls [40]. All animals were tested in a randomized order at the start and end of the experimental protocol. Testing was performed in the same room and at the same time (between 8–10 am) to ensure environmental consistency. Briefly, each animal was placed into the centre of the Y-maze and each arm entry was recorded. An entry into an arm was considered valid if all four paws entered the arm. An alternation was defined as three consecutive entries in three different arms (i.e. 1, 2, 3 or 2, 3, 1, etc). The percentage alternation score was calculated using the following formula: Total alternation number/Total number of entries $-2)$*100. Furthermore, total number of arm entries was used as a measure of general activity in the animals. The maze was wiped clean with 70% ethanol between each animal to minimize odour cues.

Statistical analysis

Student's *t*-test and ANOVA were used for statistical evaluation.

Immunohistochemistry

At the end of the study, all mice were euthanized with an overdose of pentobarbital and the brain removed. The brains were immediately dissected sagitally, with one hemisphere fixed in methacarn (methanol:chloroform:acetic acid; 6:3:1) and the other frozen in liquid nitrogen. After 16 h the fixed tissue was transferred to 70% ethanol and embedded in paraffin. Immunostaining of the 6 μm paraffin sections was performed using the peroxidase–anti-peroxidase method with DAB as the chromogen. Assessment was made either qualitatively or quantitatively by measuring the percentage area covered in the hippocampal and cortical region for Aβ and for protein-bound HNE analysis by measuring the cellular densitometric levels using image analysis software (Axiovision Rel 4.5, Zeiss) [39,41].

Dot-blot analysis

Frozen brain samples were homogenized in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH = 7.6) and protein concentrations were determined using the BCA method (Pierce). Dot blot analysis of homogenized protein samples for all mice was performed in two separate experiments and in triplicate each time. Five micrograms of each mouse brain homogenate was dotted onto Immobilon (Millipore) and dried. Following a blocking step in 10% non-fat milk, primary antibodies were incubated overnight followed by peroxidase labelled secondary antibodies and developed using enhanced chemiluminescence (Hyperfilm, Amersham). Results were scanned and densitometric values were obtained using Axiovision 4.5 (Zeiss) and expressed as the mean of all trials using the Student's *t*-test to compare groups.

Antibodies used for all studies included rabbit antisera directed against A$\beta_{1\text{-}42}$ (Biosource), heme oxygenase-1 (HO-1) [7,42], HNE [11], carboxymethyllysine [5], nitrotyrosine [43], monoclonal antibodies specific for A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$ (gift of Fukumoto, H., Takeda Chemical Industries), as well as 4G8 (Pierce-Endogen) and the assay for redox active iron [44,45].

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Results

Markers of oxidative modification but not amyloid-β load are decreased with LA

A significant decrease in the expression of HO-1 (Figure 1A) as well as protein-bound HNE (Figure 1B) was observed in both wild-type and transgenic mice treated with LA using immunoblot analysis of brain homogenates. No differences were detected in either nitrotyrosine or carboxymethyllysine levels following LA treatment (data not shown) in control or transgenic mice. In AβPP transgenic mice, the Aβ load was not significantly changed in either control or transgenic mice by the LA diet (Figure 1C).

Immunocytochemistry

Supporting the immunoblot analyses, HO-1, which is expressed specifically in the regions surrounding the amyloid plaques in the Tg2576 mice on control diet (Figure 2A), was decreased around the amyloid plaques in mice receiving LA (Figure 2C). Similarly, the LA diet decreased protein-bound HNE expression surrounding Aβ plaques (Figure 2D) in mice compared to those on control diet (Figure 2B).

Redox active iron accumulation was specifically colocalized with Aβ plaques in all Tg2576 mice on the normal diet (Figure 3B) in the hippocampus and cortical regions as well as in all mice receiving the chronic LA diet (Figure 3A). Aβ load showed no change either qualitatively or after quantitative analysis of the area immunostained in the entire cortical and hippocampal area (Figure 4). Age at commencement of study did not impact amyloid load in LA-treated animals (data not shown).

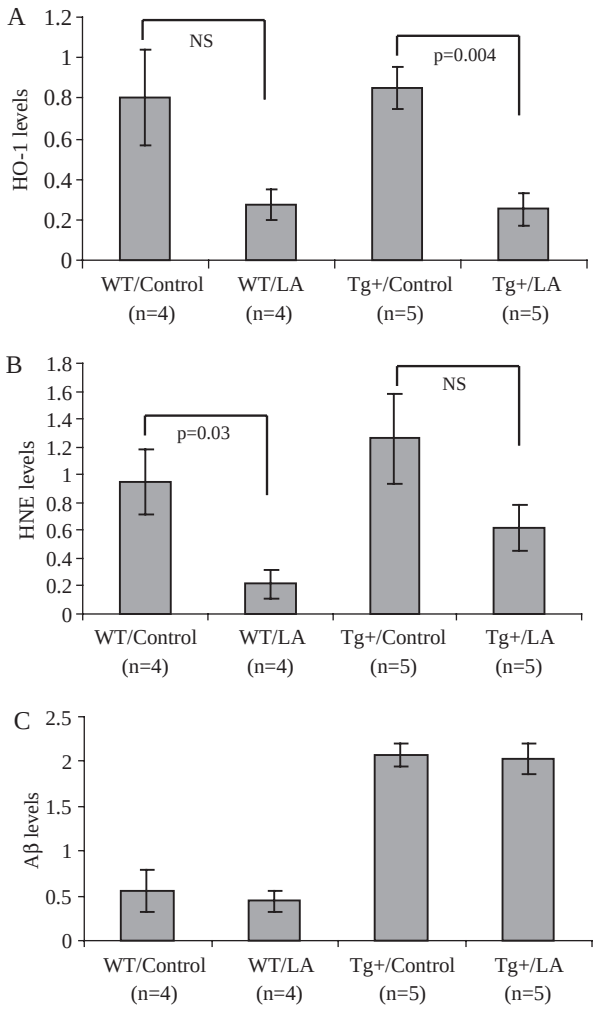

Figure 1. Dot-blot analysis of total brain homogenate reveals striking differences in markers of oxidative damage. Specifically, in both wild-type and Tg2576 mice who received the LA-enriched diet, levels of HO-1 (A) and HNE (B) were reduced, in some cases significantly. Using this method, the amount of Aβ was also determined. In the Tg2576 mice, Aβ levels remained unchanged following the chronic LA diet (C).

Lipoic acid does not alter cognition

Behavioural testing using the Y-maze was carried out at day 0 to establish a baseline measurement and at the end of the study to determine the effects of LA diet in all groups. Tg2576 mice, as expected, showed fewer spontaneous alternations, a difference which was not statistically significant using ANOVA ($p > 0.05$). At day 298, neither the control nor LA diet group showed any significant difference in alternation behaviour from the beginning of the study (Figure 5B).

Using ANOVA analysis, no significant differences ($p > 0.05$) were noted in either the number of entries or percentage alternations between the groups of mice aged 6–8 or 11–12 months of age at the beginning of the study (data not shown).

Discussion

In this study, we show that chronic administration of the antioxidant LA decreased the expression of protein and lipid peroxidation markers of oxidative modification within the brains of both control and AβPP-transgenic mice. In a related study [46], LA treatment improved Morris water maze performance in the Tg2576 mouse model, but was ineffective at modulating cognition in the Y-maze test or in the wild-type group for both tests. Significantly, the present work expands upon this by showing that administration of the antioxidant at timepoints well before the onset of plaque development and increasing the duration of treatment to 10 months neither improves cognition nor reduces amyloid deposition. Importantly, despite the small number of animals per group in our study, the Y-maze data for the control and Tg2576 mice at Day 0 is very similar to a previous study [37]. Nevertheless, we did not find differences in general activity across groups, as has been indicated previously for this task [38].

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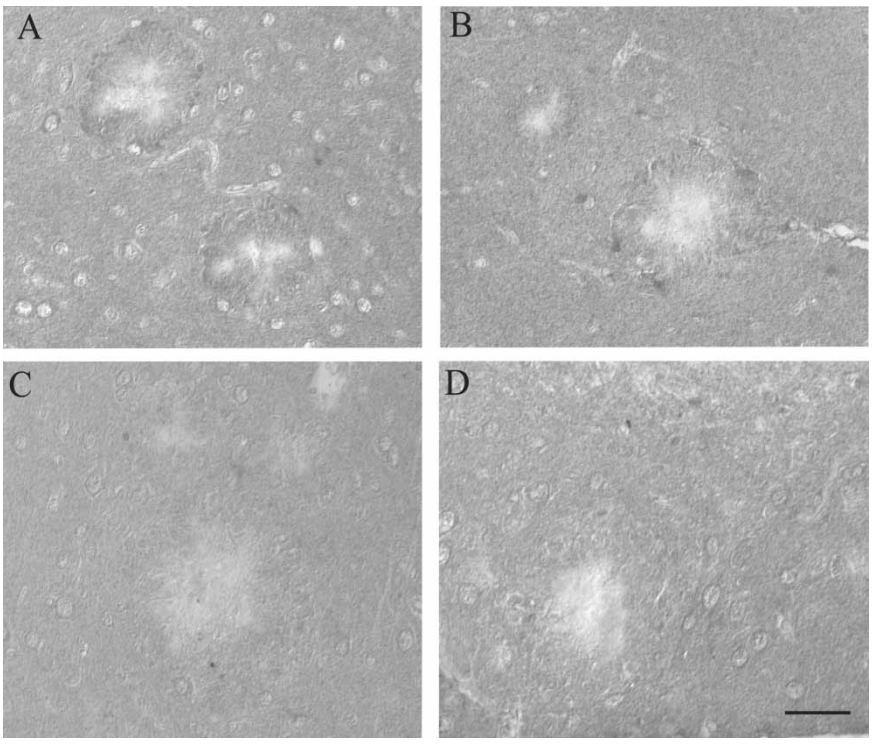

Figure 2. Immunohistochemical analysis suggests accumulation of markers of oxidative damage in the brain is attenuated following chronic LA administration. HO-1, which accumulates around the Aβ depositions in the Tg2576 mice on normal diet (A), is reduced in the mice on the LA diet (C). Similarly, in the normal diet group, HNE accumulation surrounding the large Aβ plaques is evident (B), while essentially absent from all animals in the LA group (D). Scale bar = 50 μm.

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In addition to finding no change in Aβ protein levels or plaque load in the brains of Tg2576 following long-term administration of LA, no changes in nitrotyrosine or carboxymethyllysine levels following LA treatment were detected. However, substantial decreases in both HO-1 and protein-bound HNE levels following LA were evident in both the wild-type and Tg2576 mice. Studies measuring HO-1 in AβPP transgenic mice to date have looked at immunohistochemical localization of oxidative stress markers, where there is a striking accumulation of HO-1 around amyloid deposits [47]. Yet, HO-1 is readily detectable in wild-type mice by western blot analysis [48]. Further, strong induction of HO-1, detectable with western blot analysis, is usually highest at time points less than 24 h after stress (hyperthermia, ischemia, etc.) [49,50]. Therefore, the finding in the present study, that HO-1 dot blot analysis of total brain homogenate from aged animals undergoing chronic rather than acute stress shows insignificant differences, is not unexpected. Importantly, the focal accumulation of HO-1 around the amyloid deposits (Figure 2) is similar to what has been previously reported [47]. The significance of the results presented here is that both HO-1 levels and accumulation around amyloid deposits were dramatically lowered following LA dietary supplementation. Similarly, while the levels of HNE are higher in the AβPP transgenic mice when the total brain homogenate is assayed, future studies using larger groups should show significance. Again, however, the specific localization of HNE protein adducts accumulating around

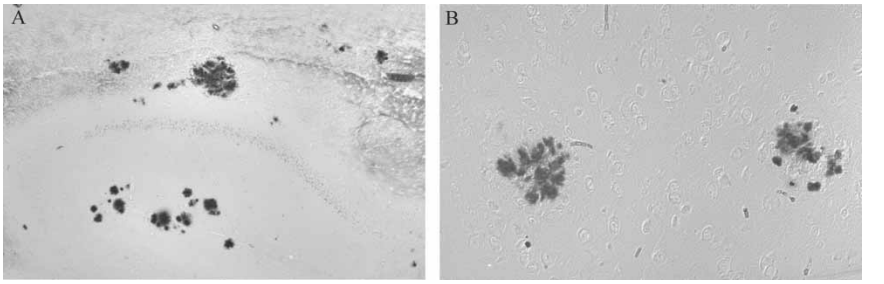

Figure 3. Redox-active iron, detected using a histochemical technique on paraffin embedded tissue sections, specifically accumulates with Aβ in Tg2576 mice on normal diet (B) and is present at the same levels even in mice on the LA diet in Aβ plaques in the hippocampus and cortex (A).

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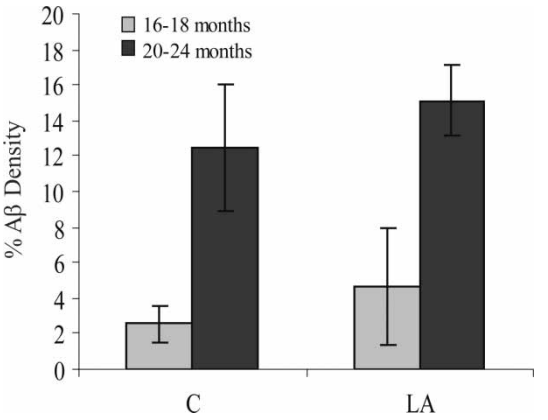

Figure 4. Immunohistochemical analysis of Aβ density in the cortex and hippocampus, expressed as the percentage area covered by Aβ, as detected with antibody 4G8 in the Tg2576 mice. While the older mice have expectedly higher levels of Aβ deposition, those mice administered the LA-enriched diet for 10 months show no less Aβ deposition. Significantly, even in those mice beginning the diet at age 6–8 months, presumably before visible Aβ plaque development, by the end of the experiment after 10 months, Aβ deposition was also not attenuated.

amyloid deposits is similar to previous reports and is also attenuated following antioxidant administration.

In the Tg2576 model, the significant development of Aβ deposits does not result in the extensive neuronal degeneration or loss as is found in AD [18,51]. The behavioural changes, which are well-documented in this model, are not associated with Aβ load [38]. The fact that Aβ deposition does not correlate well with AD severity [52] and occurs in many normal aged individuals [53,54], as well as in many other species [55,56], suggests Aβ is not a direct cause of the disease [18,57,58]. Many reports have even suggested that Aβ, as well as other fibrillar proteins in other neurodegenerative diseases, may accumulate as a protective response [59–64]. LA, however, while not decreasing Aβ deposition, has been shown to counteract the inflammation responses seen in mice following Aβ vaccination [65]. The present study, and others showing significant cognitive improvements following antioxidant therapies [66,67] yet no Aβ load attenuation, provides further evidence for the idea that amyloid is not a direct cause of the clinical manifestations of AD [68–70].

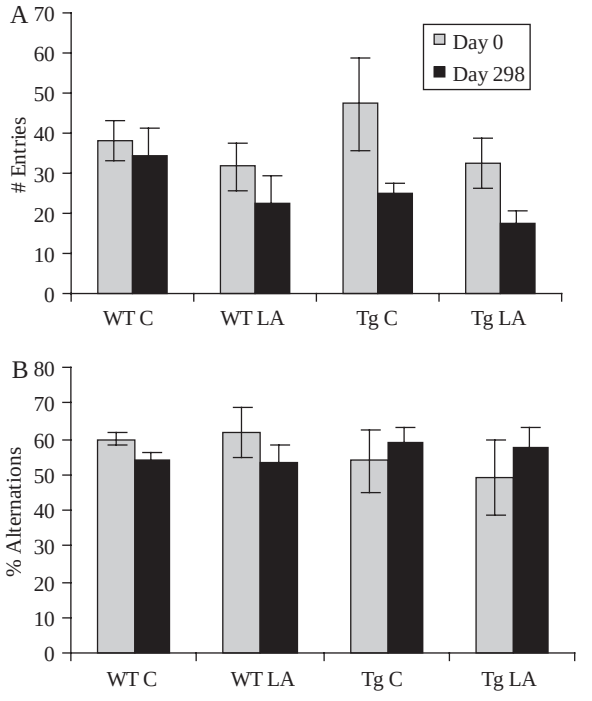

Figure 5. Behaviour analysis, in this case administration of the Y-maze task, showed little change between the groups of mice on normal or LA-enriched diet. The number of arm entries, while higher in the Tg mice at the beginning of study in accordance with previous reports, decreased by the end of the study in all groups. No significant differences were noted between the mice on normal or LA-enriched diet (A). The percentage alternations, defined as entries into an arm different than the previous two choices, did not show any significant changes in any group (B).

These studies may be applied to other non-Aβ expressing models of neurodegeneration, including the tauopathy models which display striking NFT accumulations. Increased reactive oxygen species, which are specifically localized in the NFT in AD, have also been found in the tauopathy mice [71]. In fact, some antioxidant therapies have been shown to delay the onset of the tau pathology which develops in these mice [72]. In another mouse model of neurodegeneration, using chronic systemic d-galactose exposure, treatment with LA effectively ameliorated neurodegeneration and cognitive function [73]. Further, in models of apolipoprotein E deficiency that result in intraneuronal amyloid inclusions, administration of combination antioxidant therapy both increased longevity and reduced inclusion formation in the hippocampus [74]. Antioxidants, therefore, attenuate oxidative damage in the Tg2576 mouse, in tauopathy models and in metabolic models and further work is needed to analyse the effects of LA on neurodegeneration involvement and cognitive decline in these and other models.

Restoring or maintaining the homeostatic balance between oxidative stressors and cellular responses is crucial to neuronal survival in both ageing and neurodegeneration [75]. Antioxidant therapy, either via nutritional guidelines [76] or pharmacological involvement, is often considered a low risk therapeutic strategy [77]. LA and other antioxidants have been used both in cell culture and animal models, most often showing significant and specific effects [78], though when applied to AD patients have only minor results including slowing the progression of the disease [22,79,80]. In a previous study with Tg2576 mice, administering vitamin E prior to but not after 5 months of age reduced Aβ deposition, yet the vitamin E attenuated lipid peroxidation in all groups [81]. These studies, taken together with the present work, suggest the action of LA may be

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targeting mitochondria, the most affected organelle responsible for AD development. Indeed, it has been shown that mitochondria are damaged in AD, structurally and functionally [82–84] and, therefore, antioxidants that easily penetrate not only the cell, but the mitochondria, may provide the greatest protection. To this end, compounds other than the naturally occurring antioxidants have much greater access to and provide protection to mitochondria from oxidative stressors [85,86].

The present study only augments both the safety and potential benefits of applying antioxidants long-term for healthy human ageing. Further studies using LA in conjunction with other antioxidants or at different concentrations is warranted. The one remarkable result from this long-term study is that chronic, low-dose antioxidant therapy, specifically LA in this case, is both safe and effective for lowering accumulations of oxidative stress products in both wild-type and transgenic aged mice. However, the lack of effect on cognitive decline or amyloid load by this therapy is not insignificant and provides evidence that antioxidants may be beneficial for healthy, normal ageing but should be used as a safe addition to other therapies aimed at stopping the neurodegeneration of AD.

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