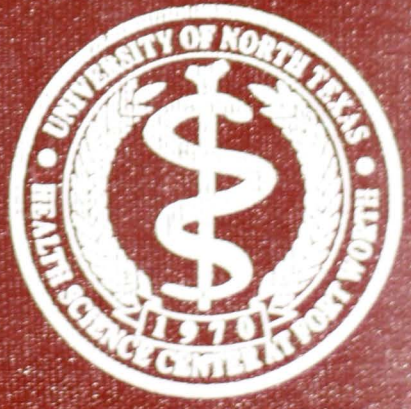
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Purpose: Aging is associated with mild to moderate loss in brain function over time. These functional losses are thought to involve reversible changes disrupting important cellular signaling processes. One of the theories that proposes to explain the reversible losses of function is the 'oxidative stress' hypothesis of aging. According to the oxidative stress hypothesis, there is an inherent cellular imbalance between production of oxidants and antioxidative defenses that increases with age and that leads to an increase in oxidative damage to macromolecules that are involved in crucial cell functions. Previous studies have established a link between these cellular changes associated with aging and the impairments in cognitive and psychomotor function. Further it has also been suggested that dietary interventions can modulate the level of oxidative stress, reducing oxidative damage and perhaps even ameliorate age-related brain dysfunction. Most interventions have been implemented relatively early in life and maintained until old age. However, the current studies were based on the rationale that interventions initiated in late-life could potentially lower oxidative damage and thereby alter cellular components responsible for functional impairments.

Methods: In study I, separate groups of young (4 months) and old mice male C57BL/6 (18 months) were fed a control diet or a diet supplemented with low (105 mg/kg/day) or high (368 mg/kg/day) concentrations of CoQ_{10} for a period of 15 weeks. After 6 weeks on the diets, the mice were subjected to a battery of age-sensitive behavioral tests. In

study II, separate groups of male C57BL/6 young mice aged 3-4 months and old mice 17-18 months (total of n=124) were fed *ad libitum* either a control diet (cyclodextrin in base diet), or the same diet supplemented with D- α-tocopheryl acetate (Toc) (200 mg/kg body wt/day), or with CoQ_{10} (148 mg/kg body wt/day) or a diet containing a combination of CoQ and Toc (200 mg/kg body wt/day + 148 mg/kg body wt/day) for a period of 13-14 weeks. In both studies mice were subjected to a battery of behavioral test that required utilization of various component of memory and learning and sensorimotor reflexes.

Results: In study I, low CoQ_{10} failed to improve cognitive and psychomotor function in old mice. However, the high CoQ_{10} marginally helped the old mice to navigate in the swim maze task with greater efficiency than control mice but did not affect their performance in probe trials. Conversely, the high CoQ_{10} diet selectively impaired the spatial performance in young mice in probe trials. The results from study I indicated that intake of CoQ_{10} initiated in late-life had minimal beneficial effects on behavior function. In study II, an age-associated decline of behavioral functioning was observed; however CoQ_{10} treatment failed to improve the performance of mice in any of the age-sensitive tests. Moreover, young mice supplemented with a high CoQ diet performed poorly in the probe trial in a swim maze task, suggesting a possible deleterious effect. The results from study II indicated that there was a significant improvement in performance of old mice in the coordinated running and the learning ability in discriminated avoidance task when supplemented with Toc or with a combination of CoQ_{10} and Toc.

Conclusions: In conclusion, these studies suggest that benefits of single antioxidant supplementation when initiated late in life are limited; however dietary supplementation

with a combination of antioxidants has a greater impact in reversing age-related decline in behavioral function.

THE EFFECT OF LATE-LIFE ANTIOXIDANT SUPPLEMENTATION

ON BRAIN FUNCTION

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CHAPTER I

INTRODUCTION

Oxidative stress theory of aging

One of the most influential modern theories of aging is known as the 'free radical theory of aging' which states that "the sum of the free radical reactions going on throughout the cells and tissues, was the aging process or a major contributor to it" (Harman, 1956). Free radicals are molecules with unpaired electrons, a state rendering them highly reactive to cellular macromolecules. It was subsequently discovered that reactive oxygen species (ROS), as well as other oxidants without unpaired electrons formed during the metabolism of molecular oxygen (O_2), can also damage cell constituents. The revised 'free radical theory of aging', now referred to as the 'oxidative stress hypothesis of aging', explains age-related loss of function as due to increased levels of ROS that damage the macromolecules crucial to normal cell functioning (Beckman & Ames, 1998; Sohal & Weindruch, 1996; Wickens, 2001).

During aerobic respiration, all cells are under a certain amount of continuous oxidative stress due to physiological production of ROS. The cellular sources of ROS include the mitochondrial electron transport chain (ETC), phagocytic cells that kill

bacteria and viruses, peroxisomes during the degradation of fatty acids and proteins, and cytochrome 450 enzymes while defending against toxins (Ames, Shigenaga, & Hagen, 1993).

Present within the cells are many natural defense mechanisms that either neutralize these free radicals or destroy the cells containing them. Some enzymes that form such a defensive system include superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and catalase. Superoxide dismutase converts superoxide ($O_2^{\bullet -}$) and hydrogen ions (H^+) to hydrogen peroxide (H_2O_2), a reaction that is facilitated by Cu-Zn SOD in the cytosol and mitochondrial inner membrane or Mn-SOD in the mitochondrial matrix (McCord & Fridovich, 1969). Hydrogen peroxide is then neutralized to oxygen and water (O_2 and H_2O) by glutathione peroxidase (GSH-px) found in the cytosol and catalase in the cytosol and peroxisomes (Beckman & Ames, 1998; Wickens, 2001). Other defensive mechanisms include endogenous non-enzymatic antioxidants: hydrophilic- vitamin C (ascorbate), urate, glutathione, and lipophilic- vitamin E (tocopherol), carotenoids, and flavonoids (Brigelius-Flohe & Traber, 1999; Halliwell & Gutteridge, 1990). Some enzymes such as dehydroascorbate reductase, thioredoxin reductase, and glucose-6-phosphate dehydrogenase are responsible for providing a reducing environment in the cell and regenerating molecular antioxidants (Beckman & Ames, 1998). However, with age, the balance between the pro-oxidants and natural defensive systems is disrupted resulting in an increase in pro-oxidants that attack macromolecules such as DNA, proteins, and lipids (Beckman & Ames, 1998;. Sohal & Weindruch, 1996; Stadtman, 1992). At various points in the electron transport chain (ETC), the increased

rate of $O_2^{\bullet-}$ and H_2O_2 production during aging occurs in parallel with an increase in the amount of oxidative damage (Sohal & Weindruch, 1996). Furthermore maximum life span (MLSP) is inversely proportional to both $O_2^{\bullet-}$/ H_2O_2 and oxidative damage accrued in the mitochondria (Barja & Herrero, 2000).

Within the biological membranes, reactive species oxidize lipids which can then attack proteins, resulting in the destruction of the catalytic action of enzymes. Oxidized proteins will lead to malfunction of channels and transporters disrupting the membrane fluidity and ultimately compromising the function of the cell (Berlett & Stadtman, 1997; E. R. Stadtman & Levine, 2000; Wickens, 2001). Membrane lipids are vulnerable to attack by ROS resulting in formation of peroxyl radical ($ROO^{\bullet}$) which can further damage other cellular components or enter a chain reaction by attacking other fatty acid side chains (Reiter, 1995). Age-related increases in lipid peroxidation have been documented, as measured by augmentations in thiobarbituric acid-reactive substance (TBARS), malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), or F_2-isoprostanes (Babusikova et al., 2007; Calabrese et al., 2004; Cini & Moretti, 1995; Gupta et al., 1991; Ward et al., 2005).

Proteins can be oxidized via many different mechanisms. One of the main type of protein oxidation is carbonylation which can occur by α-amidation pathway, by oxidation of glutamyl residues, or by direct oxidation of lysine, arginine, proline, or threonine residues. Other indirect methods of protein oxidation include direct reaction with MDA or with other reactive carbonyl derivatives (Stadtman, 1998).

Proteins and lipids, upon iron-catalyzed oxidation, also form cross-linked undegradable products called lipofuscin (Brunk & Terman, 2002). Accumulation of lipofuscin in cells results in increased sensitivity of cells to oxidative stress, leading to apoptosis (Terman & Brunk, 2004). In postmitotic cells such as neurons, cardiac myocytes, and skeletal muscle fibers, there is a continuous accumulation of lipofuscin over time that is inversely proportional to life-span (Brunk & Terman, 2002).

Similarly, ROS may cause damage to DNA, resulting in modifications on bases and sugars, strand breaks, cross-links to other nucleic acids or proteins, and base deletions and insertions (Stadtman & Levine, 2000; Wickens, 2001). A comparison of 8-hydroxydeoxyguanine (8-OHdG), a measure of DNA oxidation from mitochondrial DNA (mtDNA), and nuclear DNA (nDNA) from heart and brain in different mammalian species ranging in age from 3.5 to 46 years led to the conclusion that 8-OHdG was inversely correlated with maximum life-span (MLSP) (Barja & Herrero, 2000; Sohal, Agarwal, Agarwal, & Orr, 1995). Larger mammalian species with similar MLSP have similar metabolic potential and also a lower oxidant production in comparison to smaller mammalian species (Ku & Sohal, 1993). Another study involving five different species of dipteran flies found that the average life-span was inversely correlated with the rate of generation of free radicals in the mitochondria, cytochrome c oxidase activity, and protein carbonyl content (Sohal, Sohal, & Orr, 1995). Therefore, based on a large body of evidence, it is generally agreed that there are strong associations between ROS production, oxidative damage of macromolecules, and maximum life-span.

Mitochondria and aging

Mitochondria are called the 'power house' of a cell as they are responsible for the ATP production in the cell. During aerobic respiration, the ETC shuffling electrons undergoes a lot of oxido-reduction reactions thus increasing the chances of "leaks" and resulting in the formation of by-products, $O_2^{\bullet-}$ and H_2O_2, are potential sources for the highly reactive hydroxyl radical which cause oxidative damage to cellular components. Thus, the 'mitochondria theory of aging' has been derived from the 'free radical theory of aging' (Harman, 1981). According to this theory, the disruption of mitochondria with age is the result of a vicious cycle of reactions leading to oxidative damage of vital mitochondrial and other cellular components, ultimately resulting in loss of physiological function (Cadenas & Davies, 2000). Many studies have reported age-related increases in oxidative damage to mitochondrial DNA, proteins, and lipids, thus providing evidence supporting the 'mitochondria theory of aging'.

Cardiolipin, which constitutes about 20% of the total lipid in the inner mitochondrial membrane, is critical to mitochondrial function as it is involved in stabilizing the activity of protein complexes in the ETC (Hoch, 1992; Paradies et al., 1997). With age, there is a decrease in cardiolipin content in mitochondria, resulting in reduced cytochrome C oxidase activity. The administration of cardiolipin restored the loss in activity of cytochrome C oxidase in heart mitochondria (Paradies et al., 1997). In the housefly, a linear age-related increase in mitochondrial protein carbonyl content with age is directly proportional to H_2O_2 production in flight muscle mitochondria and inversely proportional to the life-expectancy (Sohal & Dubey, 1994). Another study from

the same laboratory reported that with age only one protein, adenine nucleotide transporter (ANT), was damaged in the flight muscle mitochondria from houseflies (Yan et al.,1997). The mtDNA is located closer to the inner mitochondrial membrane than is the nDNA and has been found to be more susceptible to oxidative damage. Studies on different species have reported that an age-associated increase in 8OHdG was higher in mtDNA than nDNA (Agarwal & Sohal, 1994; Ames et al., 1993). Therefore, mitochondria play a pivotal role in maintaining cell function with age.

Oxidative stress and age-related decline of brain function

Aging is associated with mild to moderate loss in neuropsychological functions that include memory and problem-solving abilities, sensorimotor coordination, reaction time, and motor activity. The mechanisms leading to functional declines, though unclear, are generally believed to involve cellular and molecular changes that are distinct from those observed in degenerative diseases such as Alzheimer's or Parkinson's diseases (Albert, 1997; Drachman, 1997; Gallagher & Rapp, 1997a; Morrison & Hof, 1997). Studies in nonhuman primates, as well as many rodent species, have indicated age-related changes in cognitive and psychomotor functions that are comparable to those of aging humans (Gallagher & Rapp, 1997b; Gower & Lamberty, 1993; Jucker & Ingram, 1997). The purpose of extensively studying these species is to identify specific dysfunctional neurological substrates for the losses of function (Sohal & Forster, 1998).

Although the mechanisms underlying the cognitive and psychomotor loss in function are not well understood, it is known that the brain utilizes about 20% of the

body's oxygen, and therefore is susceptible to attack by reactive oxygen species. Increased levels of ROS result in a substantial increase in oxidative damage to macromolecules in the aging rodent brain (Sohal et al., 1994; Sohal et al.,1994; R. S. Sohal, Wennberg-Kirch, et al., 1999). Thus, 'oxidative stress' has been postulated to be one of the mechanisms leading to functional declines in the brain (Beckman & Ames, 1998).

There have been numerous studies in which oxidative stress has been associated with disruptions in behavioral performance. For example, protein oxidation, measured as carbonyl content in the cerebral cortex, correlated with performance on the acquisition components of swim maze task, and bridge walking correlated with protein oxidation in the cerebellum (Forster et al., 1996). In Long Evans rats exhibiting spatial learning impairments, the immunoreactivity to 8-OHdG was significantly higher in the dentate gyrus and in the CA1 region of the hippocampus. There was also an increase in carbonyl residues and significant damage in mitochondrial DNA in the impaired rats (Nicolle et al., 2001). Aged gerbils pretreated with a spin-trapping N-tert-butyl-α-phenylnitrone (PBN) for a period of 14 days had a significant reduction in the level of oxidized proteins and increases in the level of glutamine synthase (GS) and neutral protease activities when compared to the old gerbils not treated with PBN. Moreover, old gerbils made more errors in an eight-arm radial maze than their young counterparts, and pretreatment with PBN reduced the error rate (Carney et al., 1991). When rats were treated with deprenyl, an irreversible monoamine-oxidase B inhibitor, improvement was observed in spatial memory in the Morris water maze task, an improvement that was attributed to reduction

in lipid peroxidation in the prefrontal cortex, striatum, and hippocampus (Kiray et al., 2006) Taken together, these data indicate that age-related increases in oxidative damage in the brain can play a critical role in predicting impairments in behavioral function.

Modulation of Oxidative Stress and Effects on Brain Aging

Numerous studies have addressed the possibility of a link between oxidative stress and brain aging. As discussed earlier, oxidative damage is hypothesized to impair the function of key cellular organelles such as mitochondria, triggering reactions culminating in cell damage and loss of critical brain function with age. The results of dietary interventions such as caloric restriction (CR) or antioxidant supplementation suggest that oxidative stress in the brain of old mice can be decreased relatively rapidly. Such lowering appeared to improve some aspects of the impaired behavioral performance of aged subjects, suggesting that age-related losses of brain function are partially or fully reversible, and may be caused by accumulation of oxidative damage. These findings support the hypothesis that dietary interventions can lower the steady state of oxidative stress and thus improve brain efficiency in aged individuals, even after significant aging has occurred.

Caloric restriction

Caloric restriction (CR) is an extensively studied experimental approach that is known to improve cognitive function and increase maximum and median life-span (Sohal & Weindruch, 1996; Weindruch Sohal, 1997; Weindruch Walford, 1988). Mechanisms that have been proposed to explain the positive consequences of CR include reduction in

oxidative damage, prevention of immunologic decline, stimulation of neurotrophic factors, and facilitation of synaptic plasticity with age (Djuric et al., 1992; Feuers et al., 1989; Mattson, 2000; Meydani, 1990; Weindruch et al., 1986).

Rodents have been the subjects in many caloric restriction studies. For example, mice maintained on a 40% CR beginning at the time of weaning did not exhibit any deficits in either motor coordination or in complex maze learning, unlike those deficits observed in control mice fed *ad libitum* (Ingram et al.,1987). Caloric restriction beginning at 3 months of age prevented age-related deficits in radial arm maze learning in mice (Idrobo et al.,1987). A restriction of 6 months (20% and 35% restriction) from the time of weaning improved learning of male mice in Y-maze (Wu et al., 2003). Caloric restriction retarded age-related deficits in motor coordination and avoidance learning in mice (Dubey et al., 1996a). Beneficial effects of CR were evident in aged (22-month old) mice in which CR had been initiated in mid-life (14 months of age); grip strength, coordination, spontaneous alternation, and altered responses to enclosed alleys were preserved (Means et al., 1993). Similarly, life-long caloric restriction prevented age-related deficits in the performance of rats in radial- arm maze and Morris water maze learning and memory tasks (Stewart et al.,1989).

One of the proposed mechanisms by which CR increases life-span and improves brain function is by lowering oxidative stress, resulting in decreased damage to macromolecules, which has been supported by many studies (Sohal et al., 1994). For example, long-term caloric restriction significantly reduced protein oxidation, as measured by carbonyl content in the striatum, cerebellum, midbrain, and cortex (Dubey

et al., 1996a). Moreover, oxidative damage present in the brains of old mice can also be decreased relatively rapidly following implementation of CR. Old C57BL/6 mice (15-22 month), after being maintained on an ad libitum (AL) diet from 4 months of age, exhibited a decrease in protein oxidative damage in the brain after being switched to a calorically restricted (CR) diet for a short period of 3 to 6 weeks (Dubey et al., 1996b; Forster et al., 2000).

Various markers of oxidative stress have been altered by CR intervention. For example, investigators compared levels of 8-OHdG, an indicator of oxidative DNA damage, in 8- and 27-month old C57BL/6 mice and reported an increase in 8-OHdG in old mice that was lowered by short–term CR (Sohal et al., 1994). Caloric restriction has reduced superoxide anion and hydrogen peroxide generation from the mitochondria and resulted in a decrease in protein carbonyl content in the kidney, liver, heart, and brain after 3 months of CR. In the same study CR did not affect the activity of the antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (Sohal et al., 1994).

Antioxidants

Dietary supplements are gaining considerable popularity among the American population as a result of their alleged health benefits. The sales for dietary supplementation increased by nearly 80% from 1994 to 2000 (Blendon et al., 2001). Supplements that are thought to have antioxidant properties have generated particular interest. Two of these antioxidants, Vitamin E and Coenzyme Q, target the radical species that attack and damage a number of macromolecules, and are pertinent to the current study: vitamin E and CoQ.

Vitamin E

Vitamin E is a generic term used for a family of lipid soluble compounds that includes four tocopherols (α, β,γ,δ) and four tocotrienols (α, β, γ, δ). This family is characterized by a chromanol ring and phytol side chain (Fig 1). The isomers of vitamin E differ in the position of the methyl groups on the chromanol ring and the saturation of the phytyl tail (Wang & Quinn, 1999). Vitamin E can be easily obtained through diet, with vegetable oil and nuts as some of the natural sources of this vitamin. Although vitamin E is present in so many different isoforms, α- tocopherol is the predominant form retained in the organism in significant amounts.

A binding protein responsible for the transport and metabolism of the vitamin, and with a high affinity for the α- isoform of tocopherol, is α- tocopherol transfer protein (α- TTP). In humans, a 30-35 kDa tocopherol-binding protein present in hepatocytes has

a preferential binding affinity for tocopherol isomers in the following order: $\alpha = \beta > \gamma > \delta$ (Kuhlenkamp et al.,1993; Wolf, 1994). In rats, a 16 kDa tocopherol binding protein is present in the liver and heart (Dutta el al., 1994). The α- TTP helps the incorporation of α- tocopherol into very low density lipoproteins (Behrens et al.,1982). Adipose tissue, liver, and muscle store the highest amounts of the vitamin after supplementation (Machlin, 1982). Disrupted functioning of α- TTP can lead to lysosomal accumulation of α- tocopherol and excretion, rather than accumulation in the plasma, eventually resulting in a lethal syndrome, ataxia with vitamin E deficiency (Ben Hamida et al., 1993; Traber et al., 1993).

Vitamin E is involved in a variety of physiological and biochemical functions. The lipophilic property of vitamin E influences its location within subcellular organelles and the cell membrane. The protective effect of vitamin E is attributable to its antioxidant and/ or membrane stabilizing property (Wang & Quinn, 1999). The membrane stability function is attributed to complex formation between free fatty acids and phospholipids, thus providing a detergent like action and preventing fluidity of the membrane (Urano, 1998). Fatty acids are very susceptible to auto-oxidation and result in formation of lipid peroxyl radicals that damage other macromolecules. Further vitamin E also works in tandem with another antioxidant, coenzyme Q (CoQ), to scavenge the lipid peroxyl radicals as indicated in the reactions below: vitamin E acts as a primary donor of hydrogen to the peroxyl radical and the phenoxyl radical of vitamin E ($TO^{\bullet}$) formed is reduced by CoQ. The ubisemiquinone ($CoQH^{\bullet}$) formed is reduced to ubiquinol ($CoQH_2$) by reducing equivalents supplied by the electron transport chain.

$$T\text{-}OH + LOO^{\bullet} \rightarrow toc\text{-}O^{\cdot} + LOOH$$

$$T\text{-}O^{\bullet} + CoQH_2 \rightarrow toc\text{-}OH + CoQH^{\bullet}$$

$$CoQH^{\bullet} + e^- + H^+ \rightarrow CoQH_2$$

Other than a radical scavenging role α- tocopherol also acts on many cellular targets effecting important cell-signaling systems. Non antioxidant role of α- tocopherol includes activation of protein phosphatase PP_2A, causing an inhibition of protein kinase C (PKC) and ultimately effecting many downstream signaling cascades that can affect aggregation of human platelets, a postulated mechanism of anti-atherosclerotic and anti-tumor effects of vitamin E in diseased states (Islam et al., 1997; Saldeen et al.,1999; Yoshikawa et al., 1998). This protective effect of α-tocopherol possible attained by down regulation of genes such as CD 36 and SR class A, disruption of these genes protects against atherosclerotic lesions (Azzi et al., 2002). Alpha tocopherol also downregulates monocytes/macrophages, oxidized LDL scavenger receptors and inhibits 5-lipoxygenase and cylcooxygenase (Azzi et al., 2002). The beneficial effects of α-tocopherol also achieved by downregulation of collagenases, ICAM-1 and integrins all cellular targets vital for cell adhesion (Azzi et al., 2002). Therefore, although the anti-atherosclerotic and anti-tumor effects α-tocopherol is fully not characterized its ability to alter some of the signaling molecules suggests a significant role in prevention of atherosclerosis and tumor.

The concentration of α-tocopherol in the plasma of mice reached a maximum level after 48 hrs following implementation of a vitamin E- supplemented diet (1g/kg diet). There were detectable levels of α-tocopherol in the kidney, heart, muscle, liver and brain golgi and lysosomes, and the levels increased throughout the 6-week period of their

supplementation (Zhang et al., 1996).Vitamin E supplementation for a period of 12 months in apoE –deficient mice resulted in an improvement in performance in Morris water maze (Veinbergs et al.,2000). A 13 week dietary supplementation of dl- α-tocopheryl acetate resulted in 50-60% increase of α- tocopherol in the cerebral cortex (Sumien et al., 2003). However it failed to attenuate lipid peroxidation and protein carbonyls in brain regions. Further it was concluded that administering vitamin E in mice did not benefit the age impaired mice in their cognitive or motor task but indicated a deleterious effect on their performance in a coordinated running task (Sumien et al., 2004).

Vitamin E and clinical studies

Vitamin E supplementation improved neurological disease states and also improved mental and physical function of healthy aged individuals (Casadesus, Shukitt-Hale, & Joseph, 2002). Studies suggest that oxidative stress may be a causative factor for many diseased states such as Parkinson Disease (PD), Alzheimer Disease (AD), cardiovascular, and cerebrovascular disease (Beckman & Ames, 1998). Therefore vitamin E being a potent antioxidant is used in many clinical trials to produce an expected outcome of protection or slowing of the progression of the disease. Data from Deprenyl and tocopherol antioxidant therapy of Parkinson disease (DATATOP) indicated that supplementing untreated PD patients with 2000 IU vitamin E/d did not alter the time required for each patient to reach a stage when levodopa therapy had to be started (Oakes, 1993). When the same dose of vitamin E was administered to patients with Alzheimer

disease of moderate severity with or without selegiline, it delayed the deterioration of function and thus delayed the need for institutionalization (Sano et al., 1997). Lohr and Caligiuri reviewed 12 clinical studies using vitamin E (doses from 1200 to 1600 IU/d) in treating tardive dyskinesia and concluded that 9 of the 12 studies reported some improvement in scores of abnormal involuntary movement scale ranging from 18% to 47% (Lohr & Caligiuri, 1996). A recent clinical trial trying to evaluate the long-term supplementation of vitamin E in risk of cancer, cancer death and major cardiovascular events called the Heart Outcomes Prevention Evaluation (HOPE) and the extended HOPE- the ongoing outcomes (HOPE-TOO) trial concluded that there was no difference in cancer incidence, cancer deaths and major cardiovascular events but could possibly increase the risk for heart failure (Lonn et al., 2005). Therefore based on the inconclusive data from both animal and clinical studies it is rather difficult to confirm the beneficial effects of the antioxidant vitamin E by itself.

Coenzyme Q

Coenzyme Q (CoQ) is a lipophilic quinine derivative also known as 2, 3-dimethoxy-5-methyl-6-polyprenyl-1, 4-benzoquinone is ubiquitous in nature. (Fig 2) (Battino et al., 1990; Lenaz et al., 1999). The fundamental role of CoQ is as a cofactor in the mitochondrial electron transport chain (ETC), shuttling electrons between Complex I, II and III and creating a transmembrane potential required for ATP production (Crane & Navas, 1997; Crane, 2001; Echtay et al., 2000; Fontaine et al.,1998; Turunen et al., 2004). The benzoquinone ring of the ubiquinone moiety exists in equilibrium with 3

different redox states, a fully oxidized form, ubiquinone 'Q', partially reduced form ubisemiquinone '$QH^{\bullet}$', and a fully reduced form ubiquinol 'QH_2' (Nohl et al., 2003). CoQ in its fully reduced ubiquinol form serves as an important role as a cell membrane stabilizer and also as an antioxidant. It has a unique property of not only acting as an antioxidant by scavenging free radicals but also recycling other vital antioxidants like vitamin E and ascorbate (Ernster & Dallner, 1995; Forsmark et al., 1991; Forsmark-Andree, Dallner, & Ernster, 1995; Mukai, Kikuchi, & Urano, 1990; Quiles et al., 2004; Takayanagi et al.,1980).

CoQ_9 is the predominant isoform in short-lived animals such as rodents while CoQ_{10} is predominant isoform in long-lived animals such as humans (Lass et al., 1997). Tissues with high metabolic activity like heart, kidney, liver and skeletal muscle contain high levels of CoQ_{10} (Ernster & Dallner, 1995). In sub-cellular fractions the highest concentration of CoQ_{10} is found in the inner mitochondrial membrane (Zhang et al., 1996). De novo synthesis of CoQ_{10} takes place in all tissues however the concentration may be compromised under diseased states and increased oxidative stress. Being a lipophilic molecule the uptake mechanism and distribution of CoQ is very similar to that of vitamin E. It is incorporated into chylomicrons after absorption from the intestine and distributed via the lymphatic system (Katayama & Fujita, 1972). The plasma concentration of CoQ is highly dependent on the plasma lipoproteins (Chopra et al.,1998). The large molecular weight and limited lipid and water solubility makes CoQ_{10} a difficult molecule to be supplemented, therefore the nature of the formulation is very important as it dictates the bioavailability of this molecule (Chopra & Bhagavan, 2006).

Short-term animal studies indicated that dietary CoQ supplementation increased its content in the liver, spleen, serum, brain cortex homogenates and mitochondria from mice (Matthews et al., 1998; Zhang et al.,1996). Lass and colleagues also indicated that after supplementing mice with CoQ_{10} for a period of 13 weeks, the tissue concentration of CoQ_9 and CoQ_{10} can be increased in various tissues like liver, kidney, heart, brain cortex and skeletal muscle as well as their mitochondria. Further the study also confirmed that CoQ_{10} intake also enhanced levels of α-tocopherol in the mitochondria of liver, heart, skeletal muscle and brain, thus producing a sparing/regeneration effect on α-tocopherol (Lass et al.,1999a). Another study also indicated that dietary supplementation of CoQ_{10} in rats for a period of 4 and 13 weeks increased the levels of both CoQ_9 and CoQ_{10} in homogenates of different tissues as well as their mitochondria (Kwong et al., 2002). It is also known that CoQ_{10} supplementation can prolong life-span of *Caenorhabditis elegans* mev-1 mutant by scavenging the superoxide anion and also by lowering its production (Ishii et al., 2004). Age-associated declines in complexes I and IV activities from skeletal muscle ETC were mitigated by CoQ_{10} supplementation (Sugiyama et al., 1995).

Contrary to the historical view, few studies have suggested that there could be a possible pro-oxidant effect of CoQ. In the cell membrane the ubiquinol (QH_2) moiety is oxidized to ubiquinone ($QH^{\bullet}$) while it reduces the free radicals, further this molecule may interact with lipid radicals and produce secondary radicals that all have pro-oxidant capabilities (Linnane & Eastwood, 2006; Nohl et al., 1998/10). The partially reduced form of CoQ, ubisemiquinone, can undergo auto-oxidation and generate H_2O_2 which in

turn can activate NFκB ultimately causing an induction in gene expression (Linnane & Eastwood, 2006). A recent long-term study on dietary supplementation of CoQ_{10} beginning at 3.5 months of age has indicated that in all tissues homogenates and mitochondria except the brain there was an increase in both CoQ_{10} and CoQ_9 concentrations. Further biochemical analyses also indicated that CoQ_{10} supplementation did not affect antioxidant enzyme systems, ETC complexes activity and oxidative damage (Sohal et al., 2006) Therefore in sight of the recent studies the antioxidant role of CoQ by itself is still in question.

Coenzyme Q_{10} and clinical studies

Experimental models of various neurodegenerative diseases suggest a beneficial effect of CoQ_{10} supplementation (Beal et al.,1994; Beal et al.,1998; Ferrante et al., 2002). Therefore several clinical trials are trying to investigate if CoQ_{10} supplementation would slow the progression of the diseased state. Preliminary studies have indicated that CoQ_{10} supplementation to a dose of 3000 mg/d was safe and tolerable. However, the plasma levels of CoQ_{10} reached a plateau by 2400 mg/d (Feigin et al., 1996; Ferrante et al., 2002; Shults et al., 1998; Shults, Beal et al., 2004). Data from pilot studies also indicated that a dose of 1200 mg/d of CoQ_{10} significantly improved the performance of Parkinson Disease (PD) subjects in motor tasks as reflected by a lower Unified Parkinson Disease Rating Scale (Horstink & van Engelen, 2003; Shults et al., 2002). There is an increase in lactate levels in the brain of Huntington Disease (HD) patients. A dose of 360 mg/d of CoQ administered for a period of 2-8 weeks was able to decrease the occipital cortex

lactate levels in HD patients (Koroshetz et al., 1997). A therapy of six or more months of therapy reduced symptoms associated with selected mitochondrial abnormalities including the mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (Bresolin et al., 1990; Hofman-Bang et al., 1995; Morisco, Trimarco, & Condorelli, 1993). Preliminary studies also provided evidence for beneficial effect in congestive heart failure, hypertension and ischemic heart disease (Baggio et al., 1993; Edlund et al., 1992; Khatta et al., 2000; Soja & Mortensen, 1997). The data from preliminary studies are encouraging however a decision of using CoQ_{10} as a treatment in a diseased state cannot be based from conclusions drawn from these studies, rather multi-national randomized clinical trials would help in drawing a conclusion. Some studies of a large magnitude have been inconclusive; while others are still in progress therefore the clinical efficacy of CoQ_{10} is still in question (Galpern & Cudkowicz, 2007).

Antioxidant combination therapy

Results from our laboratory indicated short-term supplementation of either of these antioxidants by themselves failed to reduce oxidative damage and improve brain function (McDonald et al., 2005). Further a long-term study on dietary supplementation of CoQ_{10} beginning at 3.5 months of age indicated that CoQ_{10} supplementation did not affect antioxidant enzyme systems, ETC complexes activity, oxidative damage, and life-span (Sohal et al., 2006) Coenzyme Q and Vitamin E are not very efficacious as antioxidants when used alone but when used in combination, they may act in synergy and provide proportionately greater protection. Reduced coenzyme Q regenerates α-

tocopherol from the tocopheroxyl radical in the mitochondria, thus playing a significant role in modulating the oxidant production (Lass & Sohal, 1998; Stoyanovsky et al., 1998). It is also known that dietary supplementation of CoQ resulted in an increase in amounts of α-tocopherol in the mitochondria and synaptosomes of various tissues (Lass et al., 1999b) suggesting a regeneration mechanism.

It is postulated that the sparing/regeneration effect between CoQ and α-tocopherol when co-administered in a diet may retard or ameliorate the age-associated decline in cognitive and/or psychomotor function. Administration of combination with α-tocopherol and CoQ improved the ability of the old mice to learn and remember the preemptive response to avoid shock in a discriminated avoidance task (McDonald et al., 2005). A combination of lipoic acid and acetyl-L-carnitine was more effective than either of the two antioxidants alone in reducing oxidative stress and restoring mitochondrial function and also prevented age-associated decline in spatial memory (Hagen et al., 2002; Liu et al., 2002; Liu et al., 2002). A 60- day supplementation with a combination of vitamin E and vitamin C improved the performance of old mice significantly in passive avoidance task (Arzi et al., 2004). The same combination of antioxidants delayed the onset need for L-dopa treatment in Parkinsons patients and also reduced the susceptibility of cerebrospinal fluid and plasma proteins to oxidative damage in Alzheimer's disease patients (Buhmann et al., 2004). Combination therapy of CoQ_{10} and vitamin E improved mitochondrial function and also stabilized the neurological function scores suggesting a slowing of the disease progression in Friedreich's ataxia patients (Cooper & Schapira, 2007).

Dietary fruits and vegetables are also a good source of antioxidants and epidemiological studies have indicated that a low intake of fruits and vegetables may increase the risk of heart disease, cataract and cancer (Ames et al., 1993). Fruits and vegetables are known to be rich in polyphenolic compounds and due to their synergistic action are potent antioxidants (Rice-Evans et al.,1995). Grape seed extract that are rich in flavonoids, a type of polyphenols; when supplemented in old male Wistar rats for a period of 30 days resulted in reduction in lipid peroxidation in different brain regions and also an increase in various mitochondrial antioxidant enzyme activity (Balu et al., 2005). Another study reported that in Fischer 344 rats supplemented with 10 % concord grape juice for a period of 3 months resulted in an increase in dopamine release from striatal slices and also an improvement in performance in the Morris water maze test. However, a supplemented with 50% grape juice in the same study resulted only in an improvement in motor performance (Shukitt-Hale et al., 2006). Proanthocyanidin, another type of polyphenolic compound present in foods such as red wine, cranberry juice and azuki beans has more antioxidative activities *in vitro* than single antioxidants like vitamin C, vitamin E and catechin (Ariga, 2004). The proanthocyanidin rich crude grape seed extract (GSE-H) had a strong anti-atherosclerotic, anti-ulcer, anti-cataract and anti-diabetic effect when compared to control animals (Ariga, 2004). Further, the crude grape extract is also known to reduce muscle fatigue after training in a human intervention study (Ariga, 2004). Cortical tissue prepared from aged rats supplemented with a diet enriched with dl-α- tocopheryl acetate and ascorbic acid resulted in an attenuation of lipid peroxide and interleukin-1β (O'Donnell & Lynch, 1998). Long-term dietary enrichment in dogs

resulted in a robust improvement in accuracy of learning after 2 years than 1 year of implementation of the diet (Milgram et al., 2005). A long term supplementation with diets with fruit and vegetable extracts having a high antioxidant activity have been reported to retard cognitive impairment and prevent age-related signal transduction deficits (Joseph et al., 1998). Thus a combination of antioxidant may provide better protection from oxidative stress than a single antioxidant and improve brain function.

Goals of the Current Research

Antioxidants like α- tocopherol and CoQ_{10} are widely used as dietary supplements among the aged population, however there is very little evidence to support the beneficial claims following the intake of these supplements. A recent study indicates that short-term intake of dl-α-tocopheryl acetate failed to produce an improvement in behavioral function and further may be detrimental when supplemented in older mice (Sumien et al., 2004). The effect of a short-term intake of CoQ10 in reversing age-related declines in behavior function has not been addressed yet. Therefore one of the proposed studies will focus on exploring the effects of single antioxidant supplementation, CoQ_{10} in old mice. It is also known that long-term supplementation involving multiple antioxidants or antioxidant rich foods has consistently yielded positive outcomes. This suggested that antioxidants would possibly work in tandem producing greater effectiveness in arresting or reversing the cellular dysfunction caused by increasing oxidative damage and delaying age-related decline in behavioral function. Preliminary data published from our laboratory has provided evidence for such an outcome (McDonald et al., 2005). Therefore the second

study will focus on studying the synergistic/or additive effect of combination of antioxidants, CoQ_{10} and Vitamin E. Thus the working hypothesis of this study is ***'Antioxidants supplemented in late-life will reverse or reduce age-related decline in cognitive and psychomotor function.'***

If the working hypothesis is correct, then either a single and/or combination of antioxidants should be more beneficial in old mice in ameliorating age- related brain dysfunction. If such is the case then this effect would be attributable to the increase in antioxidant effectiveness in the brain resulting in decrease in oxidative damage in the mitochondria. This antioxidant therapy should result in an increase in levels of the antioxidants in the mitochondria and reduce oxidative damage resulting in improvement of age-related functional decline. The project will examine different markers of oxidative damage in the mitochondria of different tissues. Thus, effectiveness of the dietary supplementation will be measured with its ability to lower the different indices of cellular and behavioral dysfunction with aging.

Fig 1: The structure of d-α-tocopherol

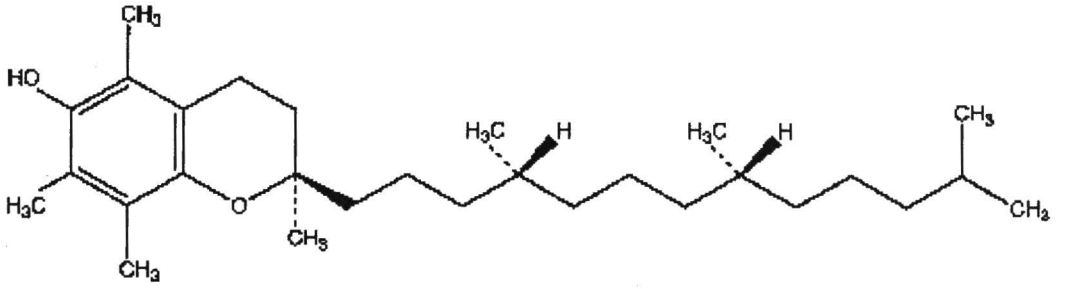
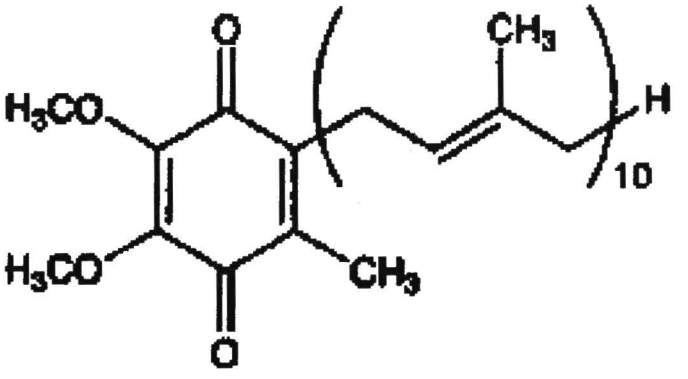

CH3
HO
H3C
O
CH3
CH3
H3C
H
H3C
H
CH3
CH2

Fig 2: The structure of ubiquinone (coenzyme Q)


O
CH3
H3CO
H
10
H3CO
CH3
O

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EFFECT OF COENZYME Q_{10} SUPPLEMENTATION IMPLEMENTED IN LATE LIFE ON AGE-RELATED COGNITIVE AND PSYCHOMOTOR IMPAIRMENTS

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CHAPTER II

EFFECT OF COENZYME Q_{10} SUPPLEMENTATION IMPLEMENTED IN LATE LIFE ON AGE-RELATED COGNITIVE AND PSYCHOMOTOR IMPAIRMENTS

Summary

Coenzyme Q (CoQ) has a number of important roles in the functioning of mitochondria and is thought to act as an antioxidant in lipid membranes. As a result, CoQ is widely available as a dietary supplement and is under investigation as a treatment for age-associated neurodegenerative diseases. However, few studies have addressed whether or not supplementation, initiated relatively late in life, could have beneficial effects on mild functional impairments associated with normal brain aging. Accordingly, the current study assessed the effect of CoQ intake in older mice for which cognitive and psychomotor impairments were already evident. Separate groups of young (4 months) and old mice (18 months) were fed a control diet or a diet supplemented with low (0.72 mg/g) or high (2.81 mg/g) concentrations of CoQ_{10} for 15 weeks. After 6 weeks, the mice were given tests for spatial maze learning, cognitive inflexibility, spontaneous locomotor activity, motor coordination, and startle reflex. Age related impairments in spatial maze learning, spontaneous rearing, motor coordination and startle reflexes were evident in the 18-month-old mice fed the control diet. The low CoQ diet failed to affect any aspect of behavioral performance in the old mice. In the test for spatial learning, both young and

old mice on the high CoQ_{10} diet could navigate to the safe platform with greater efficiency than mice on the control diet. However, neither the young nor the old mice on the high CoQ diet showed improved spatial performance as measured using probe trials and, in the young mice, this diet impaired spatial performance. Overall, the results of this study suggest that CoQ supplementation, initiated relatively late in life, has minimal beneficial effect on age-impaired cognitive or psychomotor performance.

Introduction

Coenzyme Q (CoQ) is widely available as a dietary supplement and is used by a significant number of individuals in anticipation of various health benefits. The chemical nomenclature of CoQ is 2, 3-dimethoxy-5-methyl-6-polyprenyl-1, 4-benzoquinone or ubiquinone, and it is a lipophilic quinine derivative [1, 2]. The benzoquinone ring present in CoQ is in equilibrium with three redox states: a fully oxidized form, ubiquinone 'Q', a partially reduced form ubisemiquinone '$QH^{\bullet}$', and a fully reduced form ubiquinol 'QH_2' [3]. CoQ plays vital roles in the functioning of mitochondria: 1) It serves as a component of the electron transport chain (ETC) as it shuttles electrons from Complex I, II and III; 2) It acts as a cofactor for uncoupling proteins; and 3) It plays a role in creating the transmembrane potential required for ATP production [4-8]. In addition to its roles in mitochondria, CoQ also serves as a cell membrane stabilizer and as an antioxidant. It can inhibit lipid peroxidation directly by preventing formation of lipid peroxyl radical ($LOO^{\bullet}$) and via the reducing action of QH_2, or indirectly, by recycling the α-tocopheroxyl radical back to α-tocopheroxyl, a process that also results in regeneration of ascorbate [9].

CoQ_{10} supplementation has proven to be beneficial in animal models of Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease (HD), and Parkinson's Disease (PD) [10-12]. Consequently, several clinical trials are being conducted to assess the potential benefits of CoQ_{10} in these conditions. A pilot study determined that 1200 mg/d of CoQ_{10} slowed functional decline in PD, and a dose of 360 mg/d decreased the cortical lactate levels in HD [13, 14]. CoQ is also one of several antioxidants being

considered for safety and efficacy in patients with mild to moderate Alzheimer's disease in a NIH-sponsored clinical trial [15].

Following a treatment regimen of six or more months, investigators reported reductions of the symptoms associated with selected mitochondrial abnormalities, including mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes [16-18]. Other preliminary studies have provided evidence of a beneficial effect of CoQ in congestive heart failure, hypertension, and ischemic heart disease [19-22].

It has been suggested that many processes of brain aging are reversible, based in part on evidence that aging does not involve extensive neuronal degeneration [23]. In particular, the consequences of age-related increases in oxidative stress, which have been linked to cognitive and psychomotor impairments of aging rodents [24-26], may be amenable to late life intervention with antioxidants. This possibility is suggested by the ability of caloric restriction to attenuate oxidative damage and improve behavioral indices of brain function relatively rapidly following implementation in rodents of advanced age [27, 28]. Similarly, antioxidant-rich foods appear to improve age-impaired cognitive and psychomotor performance in rodents [29].

Several investigations suggest that supplementation of CoQ_{10} could have effects similar to caloric restriction or antioxidant-rich foods. A previous study indicated that when CoQ_{10} was supplemented in the food of mice for a short period, there was an increase in content of both CoQ and α-tocopherol in the mitochondria of skeletal muscle, liver, kidney, and brain [30]. In studies performed *in vitro*, the increase in α-tocopherol produced by CoQ was inversely proportional to the generation of superoxide ($O_2^{\bullet -}$) by

mitochondria [31]. Endogenous ubiquinol in bovine submitochondrial particles inhibited both lipid peroxidation and protein oxidation [32] and, similarly, increasing the pool of ubiquinol in the mitochondria in the presence of succinate and antimycin resulted in a decrease of 8-hydroxy-deoxyguanosine and DNA strand breaks, both measures employed to assess DNA damage [9, 32-36].

While the importance of CoQ in mitochondrial function and its status as an antioxidant have led to therapeutic applications and clinical trials in neurodegenerative diseases, the potential for CoQ to ameliorate or reverse mild cognitive and psychomotor impairments associated with normal aging has not been fully evaluated. In a previous study, CoQ_{10} supplementation for three weeks prior to testing failed to yield improvement in performance of aged mice tested under a discriminated avoidance paradigm [37]. The purpose of the current studies was to provide a more comprehensive neurobehavioral evaluation of the potential effect of CoQ supplementation in aged mice. Accordingly, the current study assessed the effect of CoQ intake in older mice over a period lasting up to 15 weeks, during which the mice were given tests for spatial maze learning, cognitive inflexibility, spontaneous locomotor activity, motor coordination, and startle reflex. In these studies, CoQ10 was added to the food in either high or low concentrations, so as to target both high doses used in clinical trials for neurodegenerative disease, and relatively lower doses comparable to supplement intake by healthy individuals.

Materials and methods

Materials

Coenzyme Q_{10} (CoQ_{10}), obtained from Tischon Corp. (Westbury, NY), was added to a base rodent diet, Purina diet 5001 (Cat. Nos. 10038 and 10039, respectively, Purina Mills Test Diet, Richmond, IN). The diet was formulated such that it yielded a low concentration of CoQ_{10} (0.72 mg/g) or a high concentration of CoQ_{10} (2.81 mg/g).

Animals

Separate groups of male C57BL/6 mice were obtained from the National Institute on Aging at 3-4 months (n=24) and 17-18 months of age (n=31) and subsequently maintained in the University of North Texas Health Science Center vivarium. The mice were housed in groups of 3 or 4 in clear polycarbonate cages (28 × 17 × 12.5 cm) and had *ad libitum* access to food (standard NIH-31 diet) and water until they were assigned to a dietary condition. The ambient temperature was maintained at 23 ± 1 °C, under a 12-h light/dark cycle starting at 0600. The animals were allowed to acclimate for a two-week period, following which they were randomly assigned to one of the three treatment groups. The mice were fed *ad libitum* either a control diet (base diet) or a diet supplemented with low (0.72 mg/g) or high (2.81 mg/g) concentration of CoQ_{10}. The mice were weighed on a weekly basis, and survival was monitored throughout the study. Food and water intake was measured daily for one week prior to behavioral testing. The daily dose of CoQ_{10} that the mouse received was either 105 mg/kg/day for the low dose or 368 mg/kg/day for the high dose, and this was based on the calculations of body weight and the average food intake of a mouse in one week. Based on procedures for

estimating human and animal dose equivalents [38], the daily intake of mice under the high CoQ_{10} diet was comparable to that used in a NIH-sponsored clinical trial involving patients with mild to moderate Alzheimer's disease (2,400 mg/d; trial NCT00117403)

Neurobehavioral measures

The mice were maintained on the control and CoQ-supplemented diets for a period of 6 weeks, following which they were subjected to a series of behavioral tests. Throughout the period of testing the mice were maintained on the supplemented diet. The behavioral tests were conducted in the following order: locomotor activity, coordinated running, spatial learning and memory, auditory and shock startle, and discriminated avoidance. The testing was conducted over a period of approximately 10 weeks.

Locomotor activity

Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16), as described previously [39]. Each mouse was placed in a clear acrylic test cage (40.5 x 40.5 x 30.5 cm) that was surrounded by a metal frame lined with photocells. The test cage was enclosed in a dimly-lit, sound-attenuating chamber equipped with a fan that provided background noise (80 dB). During a 16-min period, movements in the horizontal plane, as well as a vertical plane 7.6 cm above the floor, were detected by the photocells and processed by a software program to yield different variables describing distance, vertical, and spatial components of spontaneous activity in the apparatus.

Coordinated running

Motor learning and maximum running performance were measured using an accelerating rotorod test described previously [40]. The apparatus was a motor-driven treadmill (Accuscan Instruments, Model # AIO411RRT525M) that consisted of a 3-cm diameter nylon cylinder mounted horizontally at a height of 35 cm above a padded surface. On a given trial, the mouse was placed on the cylinder, which then began rotating with increasing speed until the animal fell to a well-padded surface. Ability of the mice to improve running performance was assessed in a series of training sessions (two per day), each consisting of four trials at 10-min intervals. The training sessions continued until the running performance (the average latency to fall from the cylinder) failed to show improvement over three consecutive sessions. The treatment groups were compared for their average latency to fall on the first seven sessions, and for the final session on which each mouse had reached its maximum stable level of performance.

Startle response

The musculoskeletal startle reflex to auditory or shock stimuli of various intensities was measured using a standard testing system (SA Lab, San Diego Instruments, San Diego CA) that employed an electromagnetic force transducer. For the auditory startle test, a mouse was placed inside an acrylic cylinder and presented with a series of mixed-frequency noise bursts (0, 90, 100, 110, 120, or 140 dB). Each acoustic signal (lasting 20 ms) was presented 12 times in a counterbalanced series, for a total of 72 trials. For the shock startle test, a mouse was placed inside the same acrylic cylinder, and a series of shocks (0, 0.02, 0.04, 0.08, 0.16, 0.24, 0.32, 0.64 mA) was delivered. Each shock stimulus (100 ms in duration and scrambled across 8 inputs to the grid floor of the

acrylic cylinder) was given five times, for a total of 45 trials. The amplitude of the startle reflex was defined as the peak response to each auditory or shock stimulus within a 250-ms time window that began with the stimulus presentation [41].

Spatial learning and memory

Spatial learning and memory were measured using a swim maze test as described previously [24, 42]. On a given trial, the mouse was allowed to swim in a 120-cm diameter plastic tank filled to 34 cm from the top edge with colored water (non-toxic white paint) and maintained at 24 ± 1 °C. An escape was provided by means of a small platform (10 × 10 cm) hidden from view 1.5 cm below the surface of the water. A computerized tracking system recorded the length of the path taken by the mouse to reach the platform, as well as the swimming speed (San Diego Instruments, San Diego CA, Model # SA-3).

During a pretraining phase, the tank was covered by a black curtain to prevent pre-exposure of the mice to visual cues present outside of the tank. In this way, mice learned the motor components of swimming and climbing onto the platform without learning its location in the tank. On each trial, the mouse was placed at one end of a 10 × 65-cm (W × L) straight alley that had a platform at the other end, and allowed to swim until it reached the platform or a maximum latency of 60 s had elapsed. The mice were given four sessions of pretraining (two per day), each consisting of five trials spaced at 5-min intervals.

After pretraining, the black curtain was removed from above the tank, and the mice were tested for their ability to learn the location of the platform using spatial cues.

This testing was divided into three phases: acquisition (eight sessions with the platform in a fixed location), retention (two additional sessions after a 66-h delay interval), and reversal (four sessions with the platform at a new, fixed location). Each session consisted of five trials, at 10-min intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. Two sessions were conducted per day, separated by a period of at least 2 h, during which the mice were returned to the home cages. After the fifth trial of sessions 2, 4, 6, and 8, a probe trial was given in which the platform was submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 s, and the trial was ended when the mouse successfully located it. On this trial, spatial bias for the platform location was evaluated in terms of the percentage of time spent within 40-cm diameter annuli surrounding the platform location. A criterion was used to confirm that all mice in the study used a spatial strategy for locating the platform position in the tank. According to this inclusion criterion, the mouse had to develop a spatial bias for the platform location within 10 training sessions, as evidenced by at least 1 entry in to the previous location of the platform on the first trial of reversal (session 11). The mice that did not reach this criterion were excluded from the swim maze data analysis. Six mice in the young and 4 mice in the old group did not reach criterion in this study.

Path length (the distance taken by the mouse to reach the platform) over sessions was used as the primary measure of swim maze performance. The rate of learning was estimated by averaging the path length in the linear phase of the descending curves for both acquisition (sessions 2-4) and reversal (sessions 12-14). Maximum performance

after learning was calculated by averaging the path length of the last two sessions of acquisition (sessions 7 & 8). Further, a retention index, the average path length over sessions 9 and 10, was calculated to measure the stability of performance after a delay of 66 hrs. The path-independent swim speed was calculated by dividing distance by the latency to reach the platform.

Discriminated avoidance test

A T-maze constructed of acrylic (black for the sides and clear for the top) was utilized for the discriminated avoidance task [37, 43]. The maze was divided into three compartments: a start box (10 x 6.3 x 6 cm), a stem (17.5 x 6.3 x 6 cm), and two goal arms (14.5 x 6.3 x 6 cm), each separated by clear acrylic doors. The maze rested on a grid floor wired to deliver 0.27-mA scrambled shock to the feet.

The test consisted of two sessions separated by 24 h. On each training trial, the mouse was placed in the start box, and the start door was removed to signal the beginning of the trial. On the first trial of the first session, the mouse received shock in the first arm entered and was permitted to escape shock by running to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5 s after the opening of the start door if the mouse had not entered the correct goal arm, or immediately upon entry into the incorrect arm. In either case, the shock continued until the correct goal arm was entered or a maximum of 60 s had elapsed. Upon the mouse's entry into the correct arm, the door was closed (to prevent departure) and, after 10 s, the mouse was removed (by detaching the goal arm) and allowed to enter a holding cage for 1 min. Training in this fashion continued at 1-min

intervals until the mouse had met the criterion of a correct avoidance (defined as running directly to the correct arm within 5 s) on four of the last five training trials. The second session of avoidance training was a reversal such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous day. Ability to learn the avoidance problem was considered inversely proportional to the number of trials required to reach criterion in each of the sessions.

Statistical analysis of data

The effects of Age and Treatment on performance on the behavioral tests were assessed using two-way analyses of variance (ANOVA) with Age and Treatment as between-groups factors. The effect of the high CoQ diet was considered in a balanced ANOVA that did not include data from mice on the low CoQ diet (as this treatment was not administered to young mice in this study). Planned individual comparisons between different age groups (young vs old control) and treatment groups (i.e., low or high CoQ diet vs age-matched control) were performed using single degree of freedom F tests involving the error term from the overall ANOVA. For the swim maze data, three-way ANOVAs were performed for each dependent variable, with Sessions as the repeated measure. The alpha level was set at 0.05 for all analyses.

Results

Body Weight

There was no change in body weight in either young or old control mice from the start to the end of the study (data not shown). Paired t-tests on these data indicated that there was no significant difference between the weights ($p > 0.42$). A two-sample t-test indicated a significant difference in body weights between young and old mice at the start of the study, and this difference was maintained until the end of the study ($p < 0.001$). Supplementation with high CoQ_{10} did not affect the weight of young mice, as confirmed by a two-sample t-test ($p > 0.34$); however, there was a significant reduction in weight of the old mice by the completion of the study ($p < 0.012$).

Locomotor activity

Distance (cm), rearing (counts), center time (s), and margin time (s) were considered in the analyses of spontaneous locomotor activity (Table 1). There were age-related alterations in rearing, center time, and margin time, but not in the distance traveled. Supplementation with CoQ_{10} did not significantly alter the distance, rearing, or center time in either age group ($p > 0.4$); however, margin time of young mice supplemented with a high CoQ_{10} diet significantly increased in comparison with their age-matched controls ($p < 0.001$). A two-way ANOVA indicated significant main effects of Age ($p<0.05$) and Treatment ($p < 0.004$), as well as a significant interaction of Age and Treatment ($p < 0.007$) for margin time. A two-way ANOVA using center time or rearing as dependent variables indicated significant main effects for Age ($p<0.05$) but not for Treatment or the interaction of Age and Treatment (all *ps* >0.3).

Coordinated running

The effects of age and supplementation on the ability of the mice to reach a criterion of stable running performance are indicated in Table 1. There was an improvement in performance of both young and old mice over a period of seven sessions; however, the older mice performed worse overall than the young mice (data not shown). There was a trend towards improved performance in young mice supplemented with the high CoQ_{10} diet, but this was not statistically significant ($p > 0.12$). Supplementation also failed to improve performance of old mice. A two-way ANOVA indicated a significant main effect of Age ($p < 0.001$), but not a significant effect of Treatment or a significant interaction of Age and Treatment (all *ps* >0.204). Planned individual comparisons confirmed that the young mice were significantly different from old mice ($p < 0.001$). The young mice remained for a significantly longer period on the rotorod than the old mice as indicated by their longer latencies in Table 1.

Spatial learning and memory

The efficiency of the mice in locating the hidden platform was assessed by the average length of the path taken over the five trials of each session. The mean average path lengths of both the young and old mice decreased over sessions during the acquisition phase (data not shown). This level of performance was maintained during the retention phase and during the reversal phase when they were required to learn a new location of the platform (data not shown). Thus, all mice were able to learn to locate the hidden platform; however, the older mice traveled longer distances when compared to young mice.

Examination of the acquisition phase data suggested that CoQ_{10} supplementation resulted in improved performance of both young and old mice. In the acquisition phase, a three-way mixed-model ANOVA with Sessions as a repeated measure indicated main effects of Age and Treatment (all *ps* < 0.012), but not a significant interaction of Age and Treatment ($p > 0.703$). In the retention phase, a similar statistical analysis revealed a main effect of Age ($p < 0.04$), but neither a main effect of Treatment nor an interaction between Age and Treatment (*ps* > 0.21). In the reversal phase, a comparison of the four treatment groups failed to indicate any significant effects of Age, Treatment, or an interaction of Age and Treatment (all *ps* > 0.48).

A learning index (LI) was analyzed separately for both the acquisition and reversal phases of the swim maze task. As described in the methods, LI represents the average path length in the 'descending/learning phase' of both the acquisition and reversal phases. For the acquisition phase, the LI of older mice was greater than that of younger mice, (Fig 1 left panel), as would be consistent with the longer path lengths required for old mice to find the platform. Supplementation with CoQ_{10} seemed to lower the indices in both age groups, indicating improved performances by young and old mice. A two-way ANOVA comparing age groups (young and old) and treatment groups (control and high CoQ_{10}) indicated main effects of Age and Treatment (all *ps* < 0.041), but no interaction of those factors ($p > 0.9$). During the reversal phase, CoQ_{10} supplementation did not appear to benefit either age group, as the analysis of the reversal learning index (Fig 1 right panel) revealed a significant main effects of Age ($p > 0.034$) but not of Treatment, or their interaction (all *ps* > 0.28).

A level of maximum spatial performance was calculated by averaging the path lengths from sessions 7 and 8 (Fig-2 left panel), during which both young and old mice had reached a plateau of efficient navigation to the platform. Overall, the performance of the young mice was more efficient than that of the old mice during these sessions. Further examination of the data also suggested that there was improvement in maximum performance in both young and old mice following CoQ_{10} supplementation. Both impressions were confirmed by a two-way ANOVA which indicated significant main effects of Age and Treatment (all *ps* <0.041) but no significant interaction ($p > 0.8$).

Similarly the ability of the mice to retain the location of the platform after a 66-hr delay was evaluated using a retention index. This index was the average path length taken to find the platform in sessions 9 and 10 (Fig-2 right panel). The analysis of this measure revealed neither main effects nor an interaction (all *ps* > 0.15).

Accuracy of spatial memory was measured by conducting a probe trial as the last trial for sessions 2, 4, 6, and 8. Figure 3 illustrates the percentage of total trial time in which a mouse was within a 40-cm annulus around the target (platform) location. As might be expected, mice spent relatively little time in the annulus during the first probe trial (in session 2). In subsequent probe trials, all groups increased the time spent in the target area. However, young control mice had a stronger bias for the platform location as compared to the other three groups until session 8, at which time young mice were generally outperforming old mice. Supplementation with CoQ_{10} did not change the performance of old mice, but it did appear to hinder the young mice in learning to search within the target area (see sessions 4 & 6). A two-way ANOVA comparing four groups

(young control, young high CoQ_{10}, old control, and old high CoQ_{10}) supported these impressions with a significant main effect of Age in session 8 and an interaction between Age and Treatment in session 4 (all *ps* < 0.023); there was no significant main effect of Treatment in any session (all *ps*> 0.21). Planned individual comparisons revealed that young control mice spent more time within the 40-cm annulus than did old control mice in sessions 4 and 8 ($p< 0.043$).

The analysis of the swim speed data (Table 1) failed to yield significant main effects or an interaction (all *ps* >0.304).

Sensory reactivity

The findings summarized in Table 1 indicated that CoQ_{10} intake did not have any significant effects on sensory reactivity but clearly indicated a significant effect of age. Analyses of variance on auditory and shock startle data indicated significant main effects of Age (*ps* <0.003), reflecting age-related declines in performance, but failed to suggest effects involving supplementation or an interaction between Age and Treatment (all *ps* > 0.229). The startle responses of the aged mice to auditory stimuli were markedly diminished at all intensities when compared with the young, as were the responses of old mice to shock stimuli at most intensities (data not shown). Analyses of startle responding to the maximum shock intensity (0.64 mA) and to auditory stimuli (average response to 90- and 100-dB sounds) indicated significant effects of Age ($p< 0.001$), but did not suggest any effect of Treatment or an interaction of Age and Treatment (all *ps* >0.2).

Discriminated Avoidance

Findings from the discriminated avoidance test indicated that the number of trials

taken to reach criterion in both session 1 and session 2 did not differ with age (Table 1). Supplementation with CoQ_{10} also had no effect on the performance of young and old mice. A two-way ANOVA failed to indicate significant main effects or an interaction for either session (all *ps* > 0.2).

Discussion

In accordance with numerous other studies, an age-associated decline in psychomotor and cognitive function was evident for the mice tested in the current study [24, 39, 40, and 44]. The most interesting findings, however, were that 1) supplementation with CoQ_{10} did not improve the deficient spatial learning and psychomotor performance of the aged mice, and 2) high dose CoQ_{10} supplementation may impair spatial learning of young mice. These findings provide no basis for anticipation of beneficial effects on cognitive function when CoQ_{10} supplementation is initiated in late life. Additionally, the results suggest that high intake of CoQ_{10} may have a deleterious effect on brain function in healthy individuals.

Supplementation of CoQ_{10} (105 or 368 mg/kg/day) in aged mice for a period of 14 weeks did not improve deficient spatial learning and probe trial performance, and failed to have a significant effect on deficient psychomotor performance as measured in several different tests. It is notable; however, that the high CoQ diet did indeed increase the efficiency with which both young and old mice could navigate to the safe platform during acquisition, and also during the stable phase of performance on sessions 7 and 8 after learning of the spatial problem had occurred. This effect suggests that a high dose of CoQ_{10} may influence performance on cognitive tests, independently of the age of the

animals. The beneficial effect of high doses of CoQ_{10} would not appear to involve an enhancement of motor performance, because such an effect was not evident for swim speed or other tests of motor performance, and path length, the measure of efficiency of naviation, should be insensitive to enhanced motor performance. It is more likely that the beneficial trends in performance in swim maze acquisition reflect a facilitation of their ability to find the platform using non-spatial strategies, or perhaps simply an increase in ability to maintain a consistent heading. This argument is supported by the lack of improvement in probe trial performance among the old mice, and the presence of impairment in the probe trial performance of young mice. This pattern should seem to clearly dissociate spatial learning from the improvement in performance on acquisition and maximum spatial performance. The dissociation is most evident in the young mice, where probe trial data suggest impaired spatial learning, whereas ability to navigate to the platform is better than age-matched controls. Thus, overall, the most important observation from this study is that CoQ supplementation failed to ameliorate age-related deficits in ability of the mice to acquire spatial strategies for locating the hidden platform, which are believed to reflect age-related alterations in plasticity of hippocampal circuits [45]. The apparent lack of an effect of CoQ on deficient cognitive performance of older mice in the current study is consistent with an earlier study, involving a different cognitive test, in which old mice supplemented with even higher doses of CoQ (up to 500 mg/kg/d), also failed to show an improved learning ability [37].

An unexpected but noteworthy finding of this study was the observation of some apparently deleterious effects in young mice following supplementation with the high

dose of CoQ. These effects included a reduction in the percentage of time spent in the platform target area during the probe trial following session 4, and an increase in the time spent in the margin of the activity chambers by young mice. The probe trial measures time spent in the location of the hidden platform and, therefore, it tests the ability of a mouse to accurately locate the position of the platform using spatial clues. Although young mice on the high CoQ_{10} diet spent significantly less time in the target area than their age-matched controls in session 4, by session 8 their learning of the location of the platform was comparable to that of young mice on a control diet. Therefore, inability to accurately navigate to the platform at the end of the 'descending/learning phase' (i.e session 4) very likely indicates a delay in the acquisition of spatial learning. A similar deleterious effect did not occur in older mice on the high CoQ diet, although such an effect may not be detectable in the context of the preexisting deficit in probe trial performance.

It must be considered that increased time spent in the margin during spontaneous motor activity could reflect an increase in anxiety of the young mice receiving the high dose of CoQ. We have no independent data to refute this possibility, and therefore, this result is also consistent with detrimental consequences following high doses of CoQ_{10} in mice.

The potential for CoQ to act as a prooxidant may provide one explanation for the detrimental effect of high doses of CoQ in the current study. Auto-oxidation of ubisemiquinone results in the formation of $O_2^{\bullet -}$. Further, this superoxide anion radical may interact with lipid radicals in the cell and produce secondary radicals that all have

pro-oxidant capabilities [50, 51]. However, because there was no significant overall deleterious effect in behavior of mice supplemented with a high dose of CoQ_{10}, it cannot be concluded that CoQ_{10} is a pro-oxidant. It has been established that age-related decline in cognitive and psychomotor function is correlated with cellular changes in the rodent brain with aging [24-26]. Evidence for an increase in oxidative damage to cellular components following CoQ_{10} is lacking, which would provide the most direct evidence in support of the pro-oxidant effect of CoQ_{10}. Further studies administering higher doses and for longer supplementation periods are required to fully investigate the possible pro-oxidant effects of CoQ_{10}.

Because short-term supplementation of CoQ_{10} did not prove beneficial in either study in reversing the age-related loss in psychomotor and cognitive function, it must be considered whether supplementation of CoQ_{10} beginning at a relatively young age might be beneficial in retarding age-related decline in function. Evidence that would argue against such an outcome, however, comes from a study in which supplementation of a CoQ_{10}-enriched diet from 3.5 to 25 months had no effect on any of the antioxidant enzymes systems, level of oxidative stress, oxygen consumption, or ETC complex activity [46]. In the same study, long-term CoQ_{10} supplementation failed to reduce age-associated protein oxidation and had no effect on the GSH: GSSG ratio. Moreover, the prolonged CoQ_{10} supplementation did not affect the life-span of the mice. Notwithstanding these slight improvements in behavioral function, it is difficult to argue against the conclusion that both in short-term and long-term studies with mice, dietary CoQ10 supplementation failed to modulate the level of oxidative stress and was therefore

unable to prevent accrual or reduce oxidative damage. Additionally, short-term CoQ_{10} supplementation failed to prevent the age-related decline in behavioral function.

The ineffectiveness of CoQ_{10} in the current study may reflect poor brain bioavailability for the powder formulation used. CoQ is a very large lipophilic molecule which suggests that its bioavailability could be limited by a lack of water solubility. Water-miscible formations of CoQ appear to have enhanced bioavailability. In comparisons of solubilized (Q-Gel®) versus powder-based CoQ_{10} formulations, it was determined that the increase in plasma CoQ_{10} was much higher in the solubilized formulation than the powder [47]. Moreover, various studies in mice and rats have reported accumulation of CoQ_{10} in the brain after supplementation with a solubilized formulation of CoQ (Q-Gel®) [30, 46, 48, and 49]. The mice in the current and previous long term studies of CoQ were fed a diet enriched with a powder formulation of CoQ. Perhaps employing a solubilized formulation of the diet could insure that more of the CoQ is transported to critical sites in the brain where it would then be available to serve as an antioxidant.

Although it was surprising that short-term CoQ supplementation had so little positive effect on behavioral tasks in the current study, there is evidence that suggests that combining CoQ with another antioxidant would have a more substantial impact on behavioral performance. Recently, concurrent administration of two antioxidants, vitamin E and CoQ, in a diet improved the ability of mice to learn the discriminated avoidance task more than did single antioxidant supplementation [52]. Similarly, work in our laboratory indicated that oral administration of a combination of CoQ (120mg/kg/day)

and α-tocopherol acetate (275mg/kg/day), for a period of three weeks, resulted in a significant reduction in protein oxidation in the cortex of mice (unpublished data). These results support the notion that a combination of antioxidants could be more beneficial than a single antioxidant.

Outcomes of clinical studies investigating the potency and efficacy of CoQ in disease states have been inconsistent. Although safe doses of CoQ have been established, few preliminary studies have reported significant trends toward improvement in motor tasks, as measured by the Unified Parkinson Disease Rating Scale. Clinical trials are currently evaluating the benefits of CoQ by using a formulation with a higher dose of CoQ_{10} and also including another antioxidant, vitamin E, in the formulation [53].

In conclusion, the benefits of CoQ_{10} as a single antioxidant are debatable, and caution must be advised when recommending this antioxidant as it may expose humans to unnecessary risks. Emerging evidence suggests that a combination therapy comprised of several antioxidants may be markedly more effective than monotherapy in preventing age-related declines in behavioral function, and this possibility should be further explored.

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Figure Legends

Figure 1: Effects of age and CoQ_{10} supplementation on swim maze performance as measured by learning index (left panel- acquisition phase; right panel- reversal phase). The learning index represents an average path length (cm± S.E.) taken by a mouse to reach the platform in the acquisition phase (sessions 2-4) and the reversal phase (sessions 12-14).

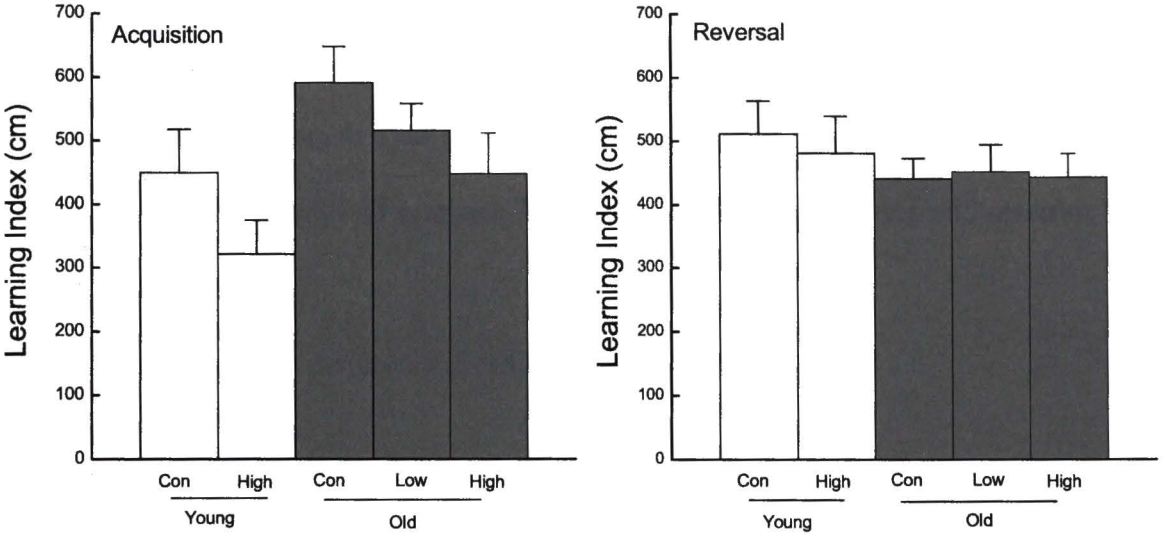
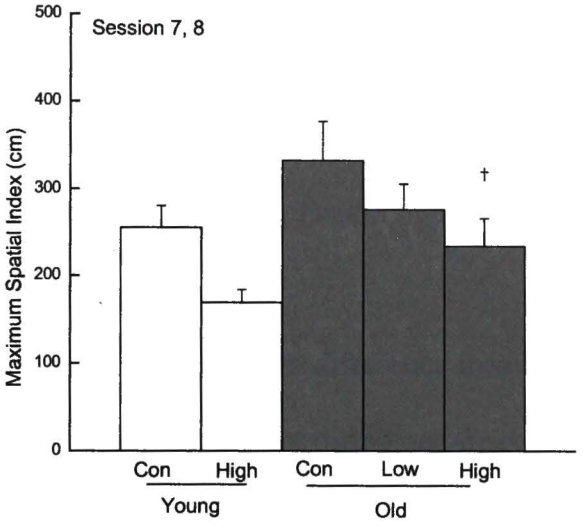

Acquisition
Learning Index (cm)
700
600
500
400
300
200
100
0
Con
High
Con
Low
High
Young
Old
Reversal
Learning Index (cm)
700
600
500
400
300
200
100
0
Con
High
Con
Low
High
Young
Old

Figure 2: Effects of age and CoQ_{10} supplementation on swim maze performance as measured by maximum spatial performance (left panel) and retention index (right panel). The learning indices represent the average path length (cm± S.E.) taken by a mouse to reach the platform (average of sessions 7-8 for maximum spatial index and sessions 9-10 for retention index).

† indicates a significant difference from age-matched controls.


Session 7, 8
Maximum Spatial Index (cm)
500
400
300
200
100
0
†
Con
High
Con
Low
High
Young
Old

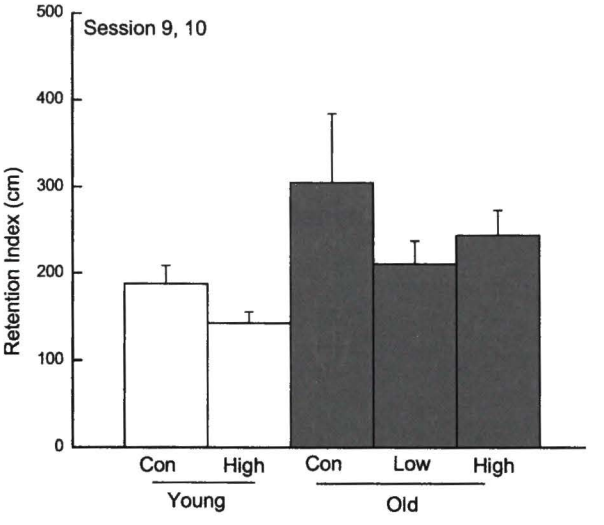

Session 9, 10
Retention Index (cm)
500
400
300
200
100
0
Con
High
Con
Low
High
Young
Old

Figure 3: Effects of age and CoQ_{10} supplementation on time spent in a 40-cm annulus during probe trials in swim maze. The percentage of time (± S.E.) spent in a 40-cm annulus surrounding the target area was calculated when platform was lowered in sessions 2, 4, 6, and 8. Dotted line indicated chance level of performance of naïve mice in the probe trial

* indicates a significant difference from young control.

† indicates a significant difference from age-matched control.

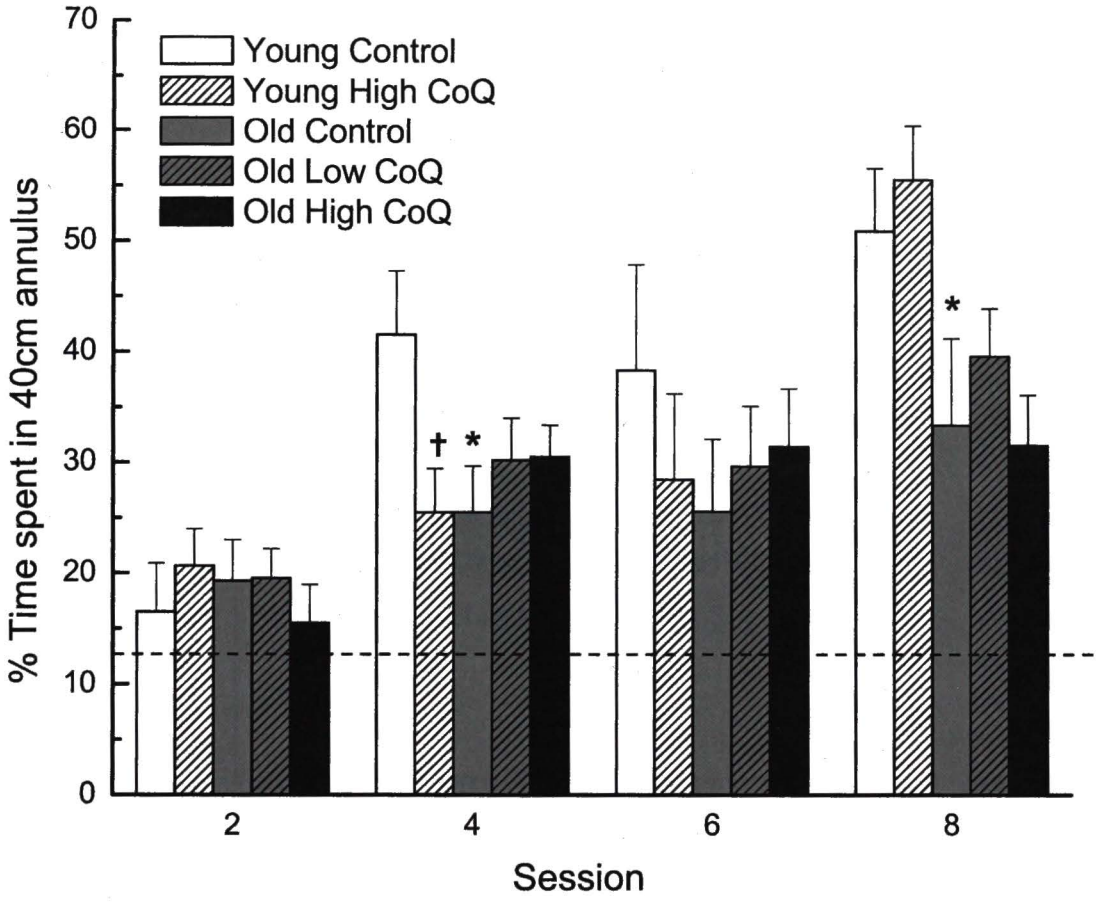

Young Control
Young High CoQ
Old Control
Old Low CoQ
Old High CoQ
% Time spent in 40cm annulus
70
60
50
40
30
20
10
0
†
*
*
2
4
6
8
Session

Table 1: Effects of age and CoQ_{10} supplementation on behavior

	YOUNG		OLD		
Behavioral Measure	Control	CoQ_{10} (H)	Control	CoQ_{10} (L)	CoQ_{10} (H)
Spontaneous Activity					
Distance (cm)	676±47	627±39	574±52	576±52	542±71
Rearing (counts)	74.7±7.0	81.4±8.1	55.5±6.6*	54.2±5.2	58.7±7.0
Center Time (s)	44.7±6.5	40.2±3.5	29.5±3.4*	41.6±5.3	40.2±3.5
Margin Time (s)	126.7±19.2	199.9±3.5†	55.8±6.6*	54.2±5.2	58.7±7.0
Reflex/Motor					
Rotorod fall (s)	51.5±2.9	60.0±7.5	32.0±2.4*	32.5±1.8	30.8±2.0
Startle (force units)					
Auditory [1]	83.3±19.6	59.2±12	22.12±3.9*	26.5±6.1	31.6±9.3
Shock[2]	666.3±78.2	577.5±65	331.7±41*	486.1±62	403.2±56
Swim Speed (cm/s)	19.1±1.09	21.2±1.6	19.0±1.3	19.4±0.7	19.6±0.9
Learning and Memory					
Swim maze					
Acquisition LI[3] (cm)	449.6±68.1	320.6±54.7	591.0±57.3	515.9±42.4	447.9±64.3
Reversal LI (cm)	511.3±52.0	481.7±57.9	441.3±32.2	452.9±42.3	443.9±37.6
Maximum Performance (cm)	255.6±25.0	169.4±14.7	332.6±45.1	276.6±29.3	233.6±32.5†
Retention index (cm)	188±20.9	142±12.9	305±79.6*	210±26.8	244±29.5
Annulus 40 (%) [4]	41.5±5.7	25.4±3.9†	25.4±4.1*	30.2±3.8	30.5±2.8
Discrim. avoidance					
Learning (trials)	18.0±1.9	18.8±2.7	19.1±1.6	18.9±1.8	15.4±2.0
Reversal (trials)	15.4±1.9	14.0±2.6	14.3±2.4	13.0±1.1	12.3±1.7

All values are the group means ± S.E.

[1] Average startle response to 90 &100 dB noise bursts

[2] Response to 0.64 mA shocks

[3] Learning index

[4] Session 4 of probe

* $p<0.05$ overall main effect of age

† $p<0.05$ overall main effect of treatment

TRANSITION REMARKS

In Chapter II, the ability of a single antioxidant, Coenzyme Q_{10} (CoQ_{10}) to ameliorate age-associated cognitive deficits was tested in aging C57BL/6 mice. The data suggested that a short-term supplementation of Coenzyme Q_{10} initiated in aged C57BL/6 mice did improve cognitive or psychomotor function. Further, the study also suggested that higher doses of CoQ_{10} could be deleterious when supplemented in young mice.

There is evidence to suggest single antioxidant supplementation in aged mice may fail to produce any beneficial, however supplementation with a diet containing multiple antioxidants or antioxidant rich foods have suggested to produce greater effectiveness amongst the aged mice population. Therefore, Chapter III deals with testing the effect of a combination diet of Coenzyme Q_{10} and α- tocopherol in aging mice. The proposed study will determine if the antioxidants Coenzyme Q_{10} and α- tocopherol have a synergistic or additive effect in restoring age-related decline in cognitive or psychomotor function.

Improvement in selected domains of cognitive and psychomotor function in old mice after supplementation of α-tocopheryl alone and in combination with Coenzyme Q_{10}

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Running title: Neurobehavioral effects of coenzyme Q and α-tocopherol

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CHAPTER III

Improvement in selected domains of cognitive and psychomotor function in old mice after supplementation of α-tocopheryl alone and in combination with Coenzyme Q_{10}

SUMMARY

The goal of the proposed study was to determine if intake of the antioxidants coenzyme Q_{10} (CoQ_{10}) or α-tocopherol (Toc), either alone or in combination, could ameliorate cognitive and psychomotor impairments of aged mice. For a period of 15 weeks, separate groups of male C57BL/6 mice aged 4 or 18 months were fed either a control diet (NIH-31 + cyclodextrin), or one of three diets supplemented with antioxidants alone or in combination (Toc, 1.65 mg/g diet; CoQ_{10}, 0.72 mg/g diet, or Toc + CoQ_{10}). After 6 weeks on the diets, the mice were administered a battery of behavioral tests designed to detect age-related impairments of cognitive and psychomotor function. Psychomotor impairments were evident in the old mice fed the control diets when they were given tests for motor coordination (rotorod), balance (bridge walking), muscle strength (wire grasp) and auditory and shock startle responses. Old mice maintained on the Toc or Toc + CoQ_{10} diets showed improved coordinated running (rotorod) performance that was equivalent to that of the young controls, whereas no improvement was evident for these groups on any other test of psychomotor function. No age-related impairment of spatial swim maze performance was evident in the old mice maintained on

the control diets in this study, although impairments were evident when these mice were tested for acquisition and reversal of a discriminated avoidance response. Mice maintained on the diet containing Toc + CoQ_{10} consistently showed improved performance in both acquisition and reversal phases of the test, to the level of the young control group, whereas CoQ_{10} and Toc alone also improved performance, albeit to a lesser degree. Overall, these results suggest that, within selected domains of cognitive and psychomotor function, antioxidant supplementation can ameliorate age-related behavioral impairment. Moreover, concurrent supplementation of CoQ_{10} and Toc may be more effective in this regard than either antioxidant alone.

Keywords: Behavior, antioxidants and mitochondria

1. Introduction

The antioxidants α- tocopherol (Toc) and coenzyme Q_{10} (CoQ_{10}) are widely used as dietary supplements among the aged population; however there is little evidence to support the anticipated health benefits. Recently, human study outcomes suggested no difference in cancer incidence, cancer deaths, or major cardiovascular events following long-term vitamin E supplementation. Moreover, these investigators also concluded that vitamin E supplementation could possibly increase the risk for heart failure (Lonn et al., 2005). Similarly, in spite of some promising preliminary studies establishing the safety of CoQ_{10} supplementation in humans, the clinical efficacy of CoQ_{10} as a single antioxidant is still in question (Galpern and Cudkowicz, 2007). Notwithstanding, both Toc and CoQ_{10} are currently the subject of clinical trails for neurodegenerative diseases of the central nervous system, including Alzheimer's disease and Parkinsonism (NCT00117403, 2006; The NINDS NET-PD Investigators, 2007).

Previous animal studies in this laboratory have indicated that when intake of Toc was initiated in later life, it not only failed to produce improvement in behavioral function, but also had deleterious in the older mice (Sumien et al., 2004a). A recent long-term study of dietary supplementation of CoQ_{10} suggested that in spite of a long-term increase of CoQ and Toc in homogenates and mitochondria of various tissues, there was no change in endogenous antioxidant defense enzymes, electron transport, oxidative damage, or life span of the mice (Sohal et al., 2006). In accordance with these findings, results from this laboratory have indicated that neither Toc nor CoQ prevent age-associated loss of cognitive function following supplementation by themselves

(McDonald and Forster, 2005; McDonald et al., 2005). Further, in a set of studies that tested for the effect of single antioxidant intake of either CoQ_{10} or Toc in old mice, there was no indication of any reduction in protein oxidation in the measured tissues (Sumien et al., 2003) or mitochondria (Sumien et al., unpublished)

Studies involving supplementation of a single antioxidant have provided inconsistent results; however, long-term supplementation involving multiple antioxidants or antioxidant rich foods has consistently yielded positive outcomes. Epidemiological studies have indicated that intake of dietary fruits and vegetables decrease the risk of heart disease, cataract and cancer. (Ames et al., 1993). Dietary fruits and vegetables are known to be rich in polyphenolic compounds, which are efficacious antioxidants (Rice-Evans et al., 1995). Old male rats supplemented with grape seed extract, for a period of 30 days, showed a reduction in age-linked lipid peroxidation in different brain regions and an increase in mitochondrial antioxidant enzymes (Balu et al., 2005). Another study (Shukitt-Hale et al., 2006) reported that Fischer 344 rats, when supplemented with different concentrations of grape juice for a period of 3 months, showed an increase in dopamine release from striatal slices and, at a relatively low concentration, an improvement of spatial performance when tested in the Morris water maze. Following relatively high concentrations, grape juice also resulted in an improvement in psychomotor function. An antioxidant rich diet containing fruit and vegetable extracts also prevented age-related decline in signal transduction and cognition (Joseph et al., 1998). Dogs supplemented with a diet enriched with antioxidants for a period of 6

months showed an attenuated age-related decline of performance in an oddity task (Milgram et al., 2002).

In addition to the apparent effectiveness of antioxidant-rich foods and antioxidant mixtures, studies of two-way combinations of specific antioxidants also suggest improved effectiveness. Lipoic acid and acetyl-L-carnitine were more effective than either of the two antioxidants alone in reducing oxidative stress, restoring mitochondrial function and preventing age-associated decline in spatial memory (Hagen et al., 2002; Liu et al., 2002; Liu et al., 2002). Similarly, vitamins E and C in combination significantly improved the performance of old mice in a passive avoidance task (Arzi et al., 2004). The same combination of antioxidants delayed the onset of need for L-dopa treatment when administered to patients with Parkinson's disease (Buhmann et al., 2004).

The findings outlined above suggest that intake of foods containing antioxidant mixtures, as well as specific antioxidants supplemented in two-way combinations, allow for physiological and perhaps synergistic interactions among the antioxidants that afford an enhanced protection against reactive oxygen species. In the current investigation, we studied the potential additive or synergistic interaction between Toc and CoQ_{10}, based on findings outlined above suggesting neither antioxidant alone was sufficient to improve or prevent age-related functional impairments. An additive or synergistic interaction between these compounds was expected based on *in vivo* and *in vitro* data suggesting a sparing/regenerative interaction between these compounds in lipid membranes. CoQ can prevent the auto-oxidation of the lipid peroxyl radical as indicated in the reactions below; vitamin E acts as a primary donor of hydrogen to the peroxyl radical, and the phenoxyl

radical of vitamin E ($TO^{\bullet}$) formed as a result is subsequently reduced by CoQ. The ubisemiquinone ($CoQH^{\bullet}$) formed is further reduced to ubiquinol ($CoQH_2$) by reducing equivalents supplied by the electron transport chain (Wang and Quinn, 1999).

$$T\text{-}OH + LOO^{\cdot} \rightarrow TO^{\bullet} + LOOH$$

$$TO^{\bullet} + CoQH_2 \rightarrow TOH + CoQH^{\cdot}$$

$$CoQH. + e^- + H^+ \rightarrow CoQH_2$$

Because reduced coenzyme Q regenerates Toc from the tocopheroxyl radical in the mitochondria, it plays a significant role in modulating the oxidant production (Stoyanovsky et al., 1995; Lass and Sohal, 1998; V et al., 1998). Supplementation of Toc, in the absence of commensurate amounts of CoQ, may limit the maximum protective effect achieved and could possibly result in excess $TO^{\bullet}$, resulting in deleterious effects.

A recent study has indicated that therapy with a combination of CoQ_{10} and vitamin E improved mitochondrial function and stabilized neurological function scores suggesting a slowing of the disease progression in Friedreich's ataxia patients (Cooper and Schapira, 2007). In accordance with these findings, a recent animal study indicated that the combination of Toc+CoQ_{10} was more effective than either compound alone, for improving the ability of the old mice to learn and remember a preemptive response to avoid shock in a discriminated avoidance task (McDonald et al., 2005). The purpose of the current studies was to provide a more comprehensive neurobehavioral evaluation of the potential effect of CoQ_{10} + Toc supplementation in aged mice. Accordingly, the current study assessed the effect of supplementing CoQ, Toc, or the combination of CoQ+Toc in old mice over a period lasting up to 13 weeks, during which the mice were

given tests for spatial maze learning, cognitive inflexibility, spontaneous locomotor activity, motor coordination, balance, muscle strength, and reaction time. In these studies, CoQ_{10} and Toc were added to the food of mice in concentrations that previously failed to result in significant improvement of age-impaired cognitive or psychomotor functions.

2. Materials and Methods

2.1. Animals

Separate groups of male C57BL/6 were obtained from the National Institute on Aging at 4 months or 18 months of age (total of n=124) and subsequently maintained in the University of North Texas Health Science Center (UNTHSC) vivarium. The mice were housed in groups of 3 or 4 in clear polycarbonate cages (28 × 17 × 12.5 cm), and had ad libitum access to food (NIH-31 diet) and water except during the testing sessions. The ambient temperature was maintained at 23 ± 1 °C, under a 12-h light/dark cycle starting at 0600. The animals were allowed to acclimate for 2-weeks before being divided into different diet groups. The mice were maintained on a supplemented diet for a period of 3 weeks following which the mice were given a series of behavioral tests. The mice were maintained on the supplemented diets throughout the period of testing. Behavioral tests were conducted on the mice in the following order: locomotor activity, simple reflexes, wire suspension, bridge-walking, coordinated running, spatial learning and memory, auditory and shock startle (sensory reactivity), and discriminated avoidance. The testing was conducted over a period of approximately 10 weeks. The mice were weighed on a weekly basis, and survival was monitored throughout the study. Food and water intake was measured daily for one week prior to behavioral testing.

2.2. Dietary supplementation

The mice were fed, *ad libitum,* either a control diet (NIH-31), or one of three diets supplemented with antioxidants alone or in combination [D- α-tocopheryl acetate (Toc), 1.65 mg/g diet; coenzyme Q_{10}(CoQ_{10}), 0.72 mg/g diet, or Toc + CoQ_{10} in the same concentrations]. The diets were formulated by Purina Mills Test Diets (Richmond, IN, Cat. Nos. 1810691, 1810688, 1810689 and 1810690 respectively). Each of the diets, including the control diet, contained 0.288% γ-cyclodextrin, which was a component of the CoQ_{10} formulation provided by Tishcon Inc. (Westbury, NY). The formulation of this compound in the diet was expected to enhance the bioavailability of CoQ_{10}. (Chopra and Bhagavan, 2006; Bhagavan and Chopra, 2007). Based on the food intake measured in the different age groups of the mice, the daily delivered doses were approximately 200 mg/kg body wt for Toc and 148 mg/kg body weight for CoQ_{10}.

2.3. Tests for Psychomotor function

2.3. 1. Locomotor activity

Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16), as described previously (Forster and Lal, 1991). Each mouse was placed in a clear acrylic test cage (40.5 x 40.5 x 30.5 cm) that was surrounded by a metal frame lined with photocells. The test cage was enclosed in a dimly-lit, sound-attenuating chamber equipped with a fan that provided background noise (80 dB). During a 16-min period, movements in the horizontal plane, as well as a vertical plane 7.6 cm above the floor, were detected by the photocells and processed by a

software program to yield different variables describing distance, vertical, and spatial components of spontaneous activity in the apparatus.

2.3.2. Simple reflex measurements

Over four consecutive daily sessions, the mice were administered three simple reflex tests. The first test consisted of placing the mouse on a flat smooth surface and recording the latency to move one body length (walk initiation). The second test measured the latency to reverse direction when the mouse was placed in a 3.5-cm wide, 14-cm long, dead-end alley (alley turning). For the third test, the mouse was placed facing downward on a flat surface that was tilted 45°, and the latency to turn 90° in either direction was measured (negative geotaxis).

2.3.3. Bridge-walking

Each mouse was tested for the latency to fall or reach a safe platform after being placed on one of four acrylic bridges, each mounted 50 cm above a padded surface. The bridges differed in diameter (small or large) and shape (round or square), providing four levels of difficulty. Each bridge was presented three times, and the measure of performance was the average latency to fall (up to a maximum of 60 s) across all bridges.

2.3.4. Wire suspension

The mouse was allowed to grip a horizontal wire with the front paws when suspended 27 cm above a padded surface. The latency to tread (reach the wire with their hind legs) and the latency to fall were recorded and averaged over four consecutive daily sessions (2 trials/ day).

2.3.5. Startle response

The musculoskeletal startle reflex to auditory or shock stimuli of various intensities was measured using a standard testing system (SA Lab, San Diego Instruments, San Diego CA) that employed an electromagnetic force transducer. For the auditory startle test, a mouse was placed inside an acrylic cylinder and presented with a series of mixed-frequency noise bursts (0, 90, 100, 110, 120, or 140 dB). Each acoustic signal (lasting 20 ms) was presented 12 times in a counterbalanced series, for a total of 72 trials. For the shock startle test, a mouse was placed inside the same acrylic cylinder, and a series of shocks (0, 0.02, 0.04, 0.08, 0.16, 0.24, 0.32, 0.64 mA) was delivered. Each shock stimulus (100 ms in duration and scrambled across 8 inputs to the grid floor of the acrylic cylinder) was given five times, for a total of 45 trials. The amplitude of the startle reflex was defined as the peak response to each auditory or shock stimulus within a 250-ms time window that began with the stimulus presentation (modified from Sumien et al., 2006) (Sumien et al., 2006). An age-related decline in amplitude of startle response to high intensity auditory stimuli occurs in C57BL/6 mice that is reflective of age-related hearing loss present in several mouse genotypes (Sumien et al., 2004a; Sumien et al., 2006). Also a measure of peak startle response following presentation of 0.64 mA shock stimulus was used in both young and old mice, is reflective of age-related sensorimotor decline

2.3.6. Coordinated running

Motor learning and maximum running performance were measured using an accelerating rotorod test described previously (Forster and Lal, 1999). The apparatus was a motor-driven treadmill (Accuscan Instruments, Model # AIO411RRT525M) that

consisted of a 3-cm diameter nylon cylinder mounted horizontally at a height of 35 cm above a padded surface. In a given trial, the mouse was placed on the cylinder, which then began rotating with increasing speed until the animal fell to a well-padded surface. Ability of the mice to improve running performance was assessed in a series of training sessions (two per day), each consisting of four trials at 10-min intervals. The training sessions continued until the running performance (the average latency to fall from the cylinder) failed to show improvement over three consecutive sessions. The treatment groups were compared for their average latency to fall on the first seven sessions, and for the final session on which each mouse had reached its maximum stable level of performance.

2.4. Tests for learning and memory

2.4.1. Swim maze

Spatial learning and memory were measured using a swim maze test as described previously (Forster et al., 1996; de Fiebre,N., Sumien,N., Forster, M.J. and de Fiebre, C., 2006). On a given trial, the mouse was allowed to swim in a 120-cm diameter plastic tank filled to 34 cm from the top edge with colored water (non-toxic white paint) and maintained at 24 ± 1 °C. An escape was provided by means of a small platform (10 × 10 cm) hidden from view 1.5 cm below the surface of the water. A computerized tracking system recorded the length of the path taken by the mouse to reach the platform, as well as the swimming speed (San Diego Instruments, San Diego CA, Model # SA-3).

During a pretraining phase, the tank was covered by a black curtain to prevent pre-exposure of the mice to visual cues present outside of the tank. In this way, mice

learned the motor components of swimming and climbing onto the platform without learning its location in the tank. On each trial, the mouse was placed at one end of a 10 × 65-cm (W × L) straight alley that had a platform at the other end, and allowed to swim until it reached the platform or a maximum latency of 60 s had elapsed. The mice were given four sessions of pretraining (two per day), each consisting of five trials spaced at 5-min intervals.

After pretraining, the black curtain was removed from above the tank, and the mice were tested for their ability to learn the location of the platform using spatial cues. Testing was divided into three phases: acquisition (eight sessions with the platform in a fixed location), retention (two additional sessions after a 66-h delay interval), and reversal (four sessions with the platform at a new, fixed location). Each session consisted of five trials, at 10-min intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. Two sessions were conducted per day, separated by a period of at least 2 h, during which the mice were returned to the home cages. After the fifth trial of sessions 8, a probe trial was given in which the platform was submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 s, and the trial was ended when the mouse successfully located it. On this trial, spatial bias for the platform location was evaluated in terms of the percentage of time spent within 40-cm diameter annuli surrounding the platform location.

A criterion was used to confirm that all mice in the study used a spatial strategy for locating the platform position in the tank. According to this inclusion criterion, the mouse had to develop a spatial bias for the platform location within 10 training sessions,

as evidenced by at least 1 entry in to the previous location of the platform on the first trial of reversal (session 11). The mice that did not reach this criterion were excluded from the swim maze data analysis. Two mice in the young and 7 mice in the old group did not reach criterion in this study.

Path length (the distance taken by the mouse to reach the platform) over sessions was used as the primary measure of swim maze performance. The rate of learning was estimated by averaging the path length in the linear phase of the descending curves for both acquisition (sessions 2-4) and reversal (sessions 12-14). Maximum performance after learning was calculated by averaging the path length of the last two sessions of acquisition (sessions 7 & 8). Further, a retention index, the average path length over sessions 9 and 10, was calculated to measure the stability of performance after a delay of 66 hrs. The path-independent swim speed was calculated by dividing distance by the latency to reach the platform.

2.4.2. Discriminated avoidance test

A T-maze constructed of acrylic (black for the sides and clear for the top) was utilized for the discriminated avoidance task (Forster and Lal, 1992a; McDonald and Forster, 2005). The maze was divided into three compartments: a start box (10 x 6.3 x 6 cm), a stem (17.5 x 6.3 x 6 cm), and two goal arms (14.5 x 6.3 x 6 cm), each separated by clear acrylic doors. The maze rested on a grid floor wired to deliver 0.27-mA scrambled shock to the feet.

The test consisted of two sessions separated by 24 h. On each training trial, the mouse was placed in the start box, and the start door was removed to signal the beginning

of the trial. On the first trial of the first session, the mouse received shock in the first arm entered and was permitted to escape shock by running to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5 s after the opening of the start door if the mouse had not entered the correct goal arm, or immediately upon entry into the incorrect arm. In either case, the shock continued until the correct goal arm was entered or a maximum of 60 s had elapsed. Upon the mouse's entry into the correct arm, the door was closed (to prevent departure) and, after 10 s, the mouse was removed (by detaching the goal arm) and allowed to enter a holding cage for 1 min. Training in this fashion continued at 1-min intervals until the mouse had met the criterion of a correct avoidance (defined as running directly to the correct arm within 5 s) on four of the last five training trials. The second session of avoidance training was a reversal such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous day. Ability to learn the avoidance problem was considered inversely proportional to the number of trials required to reach criterion in each of the sessions.

2.5. Statistical analysis of data

The effects of Age and Treatment on performance on the behavioral tests were assessed using two-way analyses of variance (ANOVA) with Age and Treatment as between-groups factors. The control diet, CoQ alone, and CoQ+Toc diet were the levels of Treatment considered in a balanced ANOVA that did not include data from mice on the Toc diet (as this treatment was not administered to young mice in this study). Planned individual comparisons between different age groups (young vs old control) and

treatment groups (i.e., each diet group vs age-matched control) were performed using single degree of freedom F tests involving the error term from the overall ANOVA. For the swim maze data, three-way ANOVAs were performed for each dependent variable, with Sessions as the repeated measure. The alpha level was set at 0.05 for all analyses.

3. Results

3.1. Body weight

There was no change in body weight in either young or old control mice from the start to the end of the study (data not shown). However, the young mice weighed less than the old mice throughout the duration of the study. None of the antioxidant diets had a significant effect on the body weight of young or old mice. A two-way ANOVA indicated a significant main effect of Age ($p < 0.001$), but not of Treatment or the interaction of Age with Treatment (all ps > 0.675).

3.2. Tests for Psychomotor function

3.2.1. Locomotor Activity

Distance (cm), rearing (counts), center time (s), and margin time (s) were selected as measures of spontaneous locomotor activity (Table 1). There were no age-related alterations in rearing, center time, and margin time, or distance traveled, and supplementation with the antioxidants alone or their combination did not significantly alter any of the measured variables in the young or old mice. A two-way ANOVA failed to indicate significant effects of Age, Treatment, or the interaction of those factors (all *ps*> 0.054).

3.3.2. Coordinated running

The effects of age and supplementation on the ability of the mice to reach a criterion of stable running performance are indicated in Fig. 1. There was an improvement in performance of both young and old mice over a period of seven sessions; however, the older mice performed worse overall than the young mice (data not shown). Supplementation with antioxidants failed to improve performance in young mice. However, when old mice were supplemented with either the Toc diet or the combination diet, their performance was improved to a level similar to that of the young mice.

A two-way ANOVA indicated significant main effects of Age and Treatment *(ps*<0.032*)* but not a significant interaction (p >0.822). However, planned individual comparisons confirmed that the young mice on the control diet performed better than the old control mice (p< 0.015) and that mice maintained on the Toc and combination diets performed better than old mice on the control diet (p<0.012).

3.2.3. Bridge-walking

Ability of the mouse to balance on a bridge was measured by the average latency to fall from 4 different bridges, each representing a differing level of difficulty (Table 1). The old mice had a tendency to fall faster from the bridges than the young mice, although antioxidant supplementations in neither young nor old mice had any effect on their latency to fall when these groups were compared with their age-matched control. A two-way ANOVA indicated a significant main effect of Age (p<0.001), but not of Treatment or the interaction of Age and Treatment (p> 0.5). Individual comparisons confirmed that

the young mice maintained on the control diet were significantly different from their older counterparts ($p > 0.001$).

3.2.4. Wire Suspension

Performance of each mouse on the wire suspension test was measured by the average over a period of 4 sessions of the latencies to fall or tread (Table 1). Young mice had a lower tendency to fall when suspended from the wire and also had a much shorter latency to tread than old mice. However, none of the antioxidant enriched diets affected the latency to tread or fall in young or old mice. A two- way ANOVA indicated a significant main effect of Age for both latency to fall and latency to tread ($ps < 0.001$), but did not indicate an effect of Treatment or an interaction of Age and Treatment ($p>0.3$). A planned comparison confirmed the effect of age in mice maintained on the control diets ($p< 0.001$)

3.2.5. Reflex battery

The reflex battery included walking initiation, alley turning and negative geotaxis, and there was no effect of either age or supplementation on the performance of the mice in these tests (Table 1). Two-way ANOVAs on data from these tests indicated neither main effects nor interactions (all $ps > 0.2$).

3.2.6. Sensory reactivity

The findings summarized in Table 1 suggest that supplementation with antioxidants alone or in combination did not have any effects on sensory reactivity but clearly indicated a decline in performance as a function of age. Analyses of variance on auditory (average response to 90- and 100-dB sounds) and shock startle (0.64 mA) data

confirmed these observations by indicating significant main effects of Age (*ps* <0.001) and failing to suggest effects involving Treatment (all *ps* > 0.384).

3.3. Tests for learning and memory

3.3.1. Swim Maze

The ability of a mouse to locate the hidden platform was assessed by the path length taken to reach the platform. Path lengths of both young and old mice decreased over sessions in the acquisition phase and reached a minimum by sessions 7 and 8. This level of performance was maintained during the retention phase and recovered to the same level of efficiency after learning of the new platform position during reversal (data not shown).

Several summary measures, each reflecting important aspects of spatial performance, were analyzed for effects of age and antioxidant treatment (Table 2). These measures included (i) a learning index (LI), reflecting average path length during the 'descending/learning phase' for both initial acquisition and reversal phases of the swim maze task; (ii) the maximum stable level of performance after learning (sessions 7 and 8); (iii) the retention index reflecting stability of performance after a 66-h delay (sessions 9 and 10); and (iv) probe trial measurements reflecting accuracy of spatial memory after session 8 (% time spent in a 40 cm annulus). There were no effects of age or antioxidant supplementation when these measures were considered in two way ANOVAs (all ps>0.12), and none of the planned individual comparisons suggested a significant difference. The analysis of the path-independent swim speed data (Table 1) failed to

yield a significant main effect of Age or Treatment and did not indicate an interaction of those factors (all *ps* >0.304).

3.3.2. Discriminated avoidance test

Two components of discriminated avoidance learning were considered for effects of age and the antioxidant treatments. Learning of the preemptive response (measured as the number of training trials needed to reach a criterion for choosing the correct goal arm within 5 sec) is shown in the top panel of Fig. 2. The discriminative component (learning to escape to the correct goal arm, measured as trials to criterion) is shown in the bottom panels of Fig. 2. For mice maintained on the control diets, there was an increase with age in the mean number of trials taken to reach the avoidance criterion for both initial acquisition (Session 1) and reversal (session 2). During session 1, all of the groups of old mice receiving antioxidant supplementation learned the avoidance response in fewer trials when compared with the old control group, and their level of learning was comparable to that of the young control group. Analysis of the trials to criterion data for session 1 revealed a significant main effect of Treatment as well as an interaction of Age and Treatment (*ps*<0.011), the latter reflecting the fact that the antioxidant treatments had effects only in the old mice. Planned comparisons indicated that performance of the old control group was significantly different from the young control group ($p<0.009$) as well as the old Toc, CoQ_{10} and Toc+CoQ_{10} groups ($p<.001$).

On session 2 (Fig. 2, top right panel) there was a similar effect of antioxidant supplementation. However, only the groups receiving the CoQ_{10} or Toc+CoQ_{10} diets differed significantly from the old control group. Analysis of the data from session 2

indicated only a significant main effect of Age (p <0.04) and did not reveal a significant Age x Treatment interaction (p>0.097). However, the planned individual comparisons indicated that the old control group differed significantly from the young control group (p<0.007) and also from the old CoQ_{10} and Toc+CoQ_{10} groups (p<0.007).

Perusal of the data for the discriminative component of the avoidance response (Fig. 2, bottom panels) failed to suggest any age-related impairment, although during session 2, there was a trend toward faster learning in the groups receiving antioxidant supplementation, relative to performance of the old control group. Analyses of the data for trials to 2 consecutive turns on sessions 1 and 2 did not indicate significant main effects or an interaction of Age and Treatment (all ps >0.160). However, individual comparisons did confirm significantly better performance in the Toc+CoQ_{10} group, when compared with the old control group (p<0.001).

4. Discussion

The main findings of the study were: 1) Both Toc and CoQ_{10} can improve performance of aged mice in a learning task, although a combination of these antioxidants was more effective than either one alone; 2) Toc, but not CoQ_{10} was sufficient to fully reverse age-related impairments measured in a test of psychomotor function; 3) The beneficial effects of the antioxidant supplementation were not generalized, but instead involved performance within specific domains of cognitive and psychomotor function.

The results of this study revealed a robust age-related decline in the ability of the control groups to learn the preemptive component of a discriminated avoidance task

during the initial session, and in the ability to reverse the direction of responding during the second session. These findings are in accordance with several previous investigations (Forster and Lal, 1992b; McDonald et al., 2005). In the current study, the antioxidants by themselves or in combination improved the performance of old mice in the initial acquisition of the discriminated avoidance task. However, the degree of improvement was most pronounced for mice fed the CoQ+Toc diet.

The second session of the discriminated avoidance task involved a reversal where the mouse learned to run to the goal arm that was opposite to the one learned in the previous session. Based on this response requirement, the impairments evident in the old mice on the control diet were likely reflective of a cognitive inflexibility, or perseveration, that can be linked to impaired frontal cortical function (Schoenbaum et al., 2006). When supplemented with either CoQ_{10} or the combination of Toc+CoQ_{10}, the old mice were improved in their ability to reverse their goal arm choice on session two. Although data for both of the discriminated avoidance sessions indicated significant improvements resulting from supplementation of a single antioxidant, it is noteworthy that a combination of the two antioxidants yielded the most robust effects. The latter finding is consistent with an additive or synergistic interaction between Toc and CoQ when supplemented in combination.

Based on the underlying rationale for antioxidant supplementation, the effectiveness of the Toc, CoQ_{10}, and Toc+CoQ10 diets for improving learning should be proportional to their ability to attenuate oxidative stress and decrease the steady-state amounts of oxidative damage in regions of the brain relevant to the behavioral functions

under study. Recent unpublished data from this laboratory appear to confirm this expectation. After only 3 weeks of daily intake of a combination of CoQ_{10}+Toc (120 mg/kg/day and 275 mg/kg/day) there was a significant reduction in protein carbonyls in the cortex of mice. Toc and CoQ_{10} supplemented by themselves were less effective in decreasing protein carbonyls in this region. These findings provide a further confirmation that Toc and CoQ_{10} act in an additive or synergistic fashion and can more effectively diminish the amount of oxidative damage.

The exact mechanism of the cooperative effect of the combination of antioxidants is not clear. However, there is considerable evidence suggesting that Toc and CoQ_{10} work in tandem to provide greater antioxidant potential. Ubiquinol regenerates Toc from the tocopheroxyl radical in the mitochondria, and the level of Toc is inversely proportional to superoxide production (Stoyanovsky et al., 1995; Lass and Sohal, 1998; V et al., 1998). Lass and colleagues (1999) concluded that only supplementation of the combination of Toc and CoQ increased the levels of Toc in the brain mitochondria, whereas such increases in the brain were not found when Toc or CoQ_{10} was supplemented alone.

While the antioxidant diets improved performance of the old mice when they were tested for discriminated avoidance learning, there was no indication of any improvement when the mice were tested for spatial learning and memory using the swim maze task. This result was not expected in light of previous studies suggesting an association between increased oxidative damage in the cerebral cortex and age-related deficits in spatial swim maze performance (Forster et al., 1996). Our unpublished finding of a reduction in protein oxidation in the cerebral cortex following CoQ_{10}+Toc, in the

absence of a benefical effect on swim maze performance in the current study, would indeed seem to disconfirm the hypothesis that oxidative stress/damage represents a proximal cause of impaired swim maze performance. On the other hand, the current results could also indicate that cellular dysfunction elicited by reactive oxygen species is not readily reversible upon attenuation of oxidative stress.

Interpretation of the lack of effect of the antioxidants on swim maze performance in this study is complicated by the finding that no age-related impairment of spatial learning or memory was evident in mice maintained on the control diets. Previous studies of C57BL/6 mice of the same age, involving both large and small sample sizes, have consistently revealed an age related impairment of spatial learning ability. Regardless of the possible reasons for the discrepant finding in the current study, it may be unreasonable to expect an improvement in swim maze performance that is already equivalent to that of young controls, even in the context of a lowering of oxidative damage.

While the current studies clearly indicate beneficial effects of combining Toc and CoQ_{10}, the apparent effectiveness of supplementing these compounds individually was not expected. In a previous study of discriminated avoidance learning, neither CoQ_{10} nor Toc was sufficient to improve performance when administered to mice in similar amounts (McDonald et al., 2005). Another unexpected finding of this study was the significant improvement in the coordinated running ability of old mice administered either Toc or CoQ_{10}+Toc, when compared to their age-matched controls. This pattern suggested that improved coordinated running could be attributed to Toc supplementation

alone, with no contribution of CoQ_{10} to the effect. This finding for Toc differed qualitatively from that reported previously. In one study, neither Toc nor CoQ_{10}+Toc yielded a benefit, while yet another suggested that supplementation with Toc in aging mice was deleterious (Sumien et al., 2004b; McDonald et al., 2005).

One explanation of the different outcomes for single antioxidants in the current and previous studies could involve a difference in formulation of the diets. Coenzyme Q_{10} used in this study was provided by Tischon Inc. (Westbury, NY) as a formulation that included cyclodextrin to enhance water solubility. Equivalent amounts of cyclodextrin were added to the control and Toc diets to equate this additive among all diets. Huang and colleagues have established that the water solubility of a highly lipophilic antioxidant like Toc can be dramatically increased by adding a 7% randomly methylated β-cylcodextrin (Huang et al., 2002). Structurally cyclodextrins are cyclic (α- 1, 4) –linked oligosaccharides of α-D-gluco-pyranose containing a hydrophobic cavity and hydrophilic outer surface. Cylcodextrins are biologically inert, well tolerated compounds and are therefore widely used in pharmaceutical industry as additives and drug-complexing agents (Szente et al., 1998; Pfitzner et al., 2000). The doughnut shaped inner cavity of cyclodextrin molecule can host large hydrophobic molecules like Toc and render them more water soluble. Another study suggested that complexation with cyclodextrins improved the antioxidant activity of flavonols in reducing malondialdehyde formation in rat liver microsomes (Calabro et al., 2004). Thus, the current results may suggest that antioxidant activity of Toc is significantly enhanced when combined with cyclodextrin. It may be particularly noteworthy that improvement of psychomotor function has not

previously been reported in the context of a single antioxidant supplemented for a short period in aged animals.

An important observation in this investigation was that the beneficial effects of antioxidant supplementation did not generalize to all behavioral tests within the domains of cognitive and psychomotor performance. Whereas supplementation of Toc was sufficient to improve coordinated running performance, this treatment did not reverse age-related impairments detected by other motor and reflexive tasks. While it has been established that bridge, wire suspension, startle reflex and rotorod tests have similar sensitivity in detecting psychomotor deficits with age, the current study results add to evidence that these behavioral tests are independently sensitive to different brain aging processes (Sumien et al., 2006). The cerebellum, motor cortex and striatum are involved in learning and coordination as measured in the rotorod task (Altman & Bayer, 1997), though this task also requires elements similar to other psychomotor tests such as appropriate visual input and muscle strength. Notwithstanding, Sumien and colleagues (2006) reported that performance of old mice on the rotorod test was not correlated with performance on any of the other tests used in the current study (Sumien et al., 2006).

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Figure Legends

Figure 1: Effects of age and antioxidant supplementation on rotorod performance as measured by latency to fall. The latency to fall represents maximum performance attained by a mouse over seven sessions (± SE)

* indicates a significant difference from young control.

† indicates a significant difference from age-matched control.

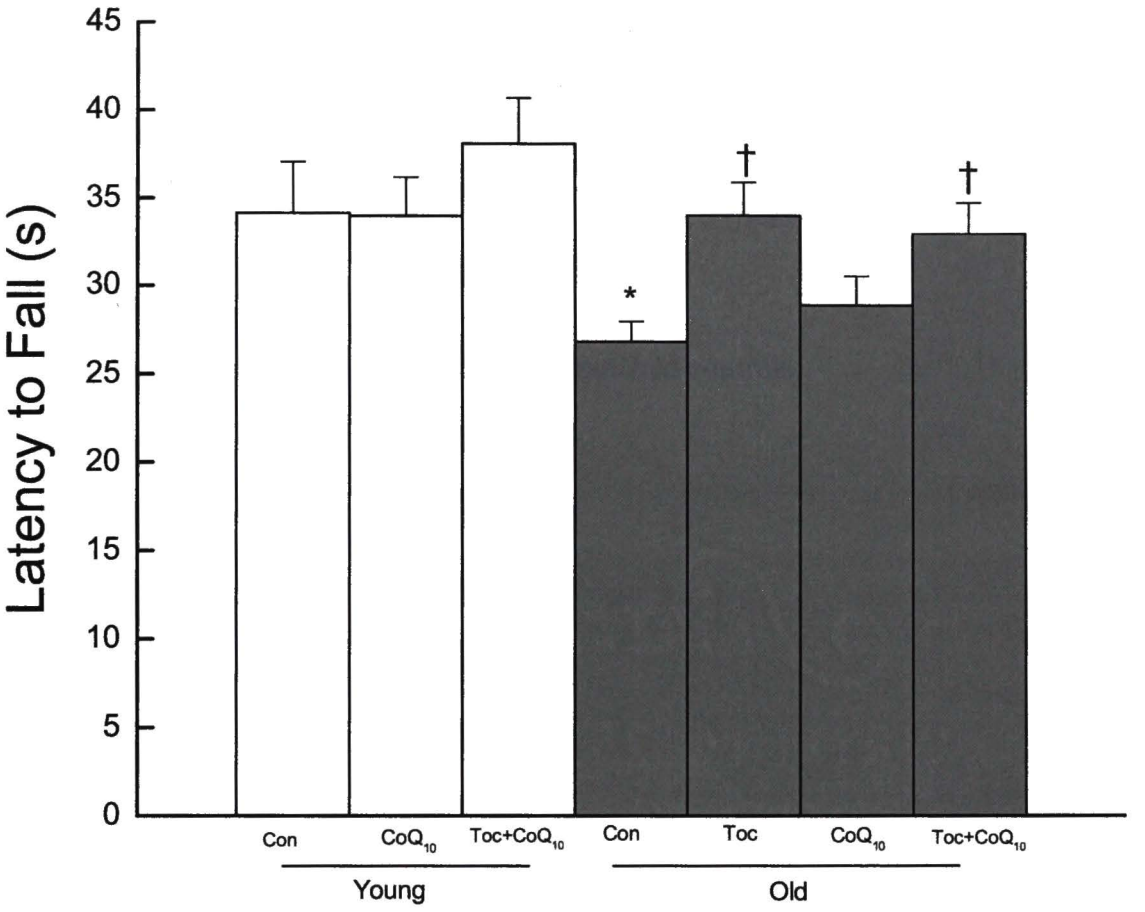

Latency to Fall (s)
45
40
35
30
25
20
15
10
5
0
*
†
†
Con
CoQ10
Toc+CoQ10
Con
Toc
CoQ10
Toc+CoQ10
Young
Old

Figure 2: Effects of age and antioxidant supplementation on discriminated avoidance task as measured by total trials (cm± S.E.) to reach criterion on day 1 (top left panel) and total trials to reach criterion on day 2 (top right panel). The bottom panel indicates total trials (± S.E.) to two consecutive correct turns for day 1 and 2 (bottom left and bottom right panel respectively).

† indicates a significant difference from age-matched controls.

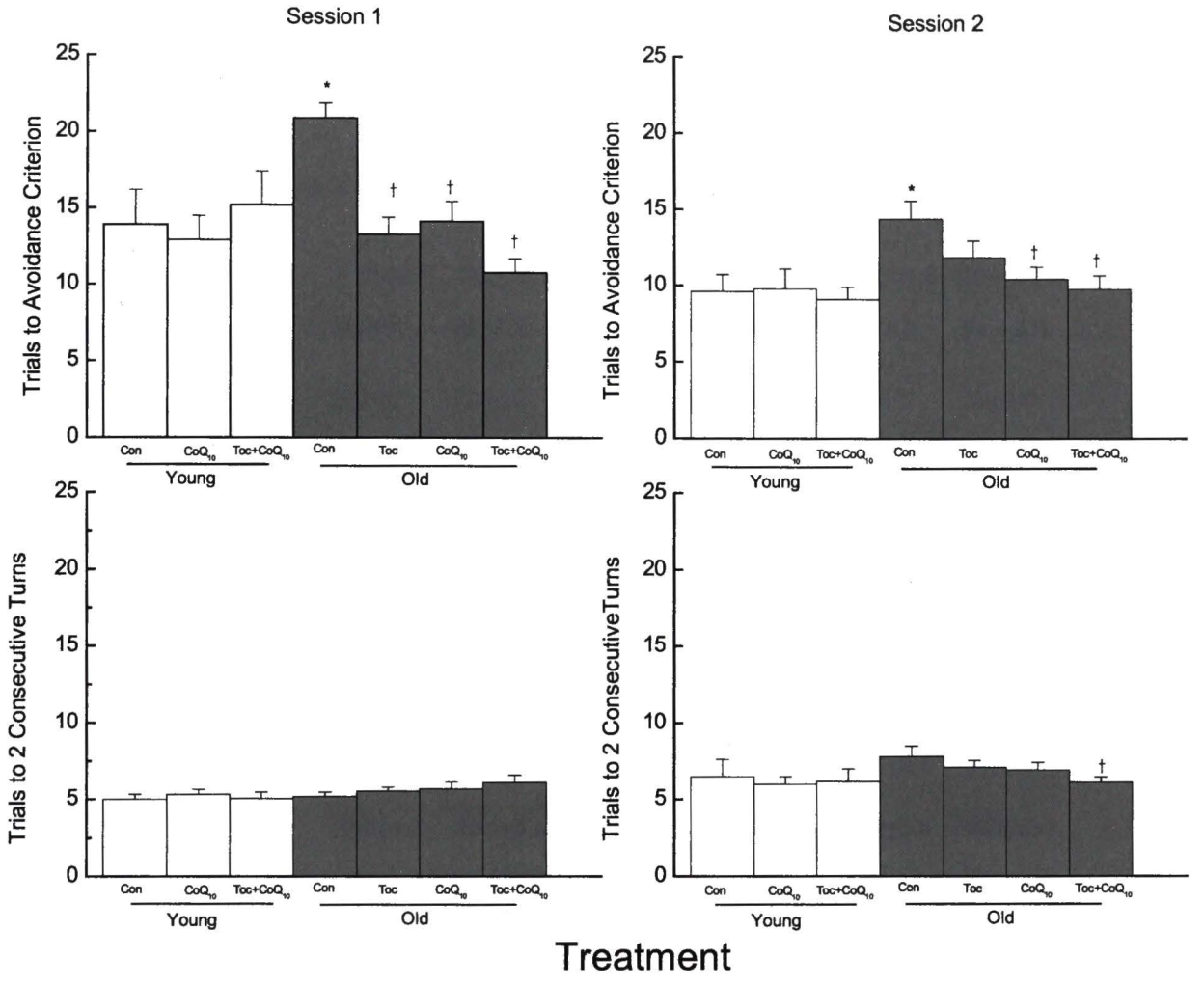

Session 1
Session 2
Trials to Avoidance Criterion
Trials to 2 Consecutive Turns
0
5
10
15
20
25
*
†
Con
CoQ10
Toc+CoQ10
Toc
Young
Old
Treatment

Table 1: Effects of age and/or antioxidant supplementation on psychomotor function

	Young			Old			
	Control	CoQ	Toc+CoQ	Control	Toc	CoQ	Toc +CoQ
Spontaneous Activity							
Distance (cm)	678±77.6	676±53.8	705±58.7	679±68.8	596±65.0	691±54.8	664±66.4
Rearing (counts)	62± 6.7	72±9.9	64±9.2	78 ± 4.3	70±8.0	62± 5.9	64 ± 5.9
Center Time (s)	35 ±3.2	36± 4.1	22±2.2	28±3.1	30± 4.2	32±3.5	38±9.6
Margin Time (s)	173±13.2	170±13.4	185±13.4	181±10.4	180±10.2	181±9.3	176±12.6
Reflex/Motor							
Walk initiation (s)	2.0±0.3	1.8±0.3	1.6±0.3	2.2±0.3	1.5±0.2	1.8±0.2	2.1±0.2
Alley Turn (s)	23.5±3.6	12.8±1.9	17.6±2.4	21.6±2	21.4±2.6	17.6±1.6	24.6±2.5
(-) Geotaxis (s)	9.5±1.3	11.4±1.6	9.9±1.7	11.6±1.3	11.5±1.1	12.4±1.3	9.8±1.1
Bridge fall (s)	48.0±3.4	48.9±1.3	51.5±1.8	29.9±2.8*	34.8±2.2	34.5±2.8	33.0±2.5
Wire fall (s)	17.6±4.7	22.9±4.2	20.5±5.2	40.3±4.4*	42.1±3.7	46.9±3.4	46.2±2.4
Wire tread (s)	17.6±4.7	22.9±4.2	20.5±5.2	40.3±4.4*	42.1±3.7	46.9±3.4	46.2±2.4
Rotorod (s)	34.1±2.9	34±2.2	38.1±2.6	26.8±1.1*	34±1.9†	28.9±1.6	33±1.7†
Startle (force units)	97.2±11.8	107±16.0	107±10.7	38.6±6.6*	35.7±8.0	42.2±4.8	51.5±5.8
Auditory[1]							
Shock[2]	724+79.2	782+66.8	749+60.0	596+58.4*	560+71.7	527+57.0	455+36.6
Swim Speed	17.4±0.9	16.5±0.6	16.3±0.7	16.9±0.5	17.6±0.5	16.8±0.6	17.6±0.3

All values are the group means ± S.E.

[1] Average startle response to 90 &100 dB noise bursts

[2] Response to 0.64 mA shocks

* $p<0.05$ overall main effect of age

† $p<0.05$ overall main effect of treatment

Table 2: Effects of age and/or antioxidant supplementation on cognitive function

	Young			Old			
	Control	CoQ	Toc+CoQ	Control	Toc	CoQ	Toc+CoQ
Learning and Memory							
Swim Maze							
Acquisition LI[1] (cm)	544±47.8	497±64.4	436±33.6	569±31.3	555±44.5	583±44.6	579±37.3
Reversal LI (cm)	384±72.6	433±96.8	348±47.2	332±21.5	337±36.8	360±49.5	396±28.8
Maximum Performance (cm)	326±62.0	266±48.0	336±54.1	393±31.9	365±35.5	323±33.6	325±36.8
Retention Index (cm)	268±39.9	227±28.3	217±24.9	290±22.0	312±34.4	332±31.8	327±37.0
Annulus 40 (%)[2]	29±5.0	23±5.4	30±5.4	23±3.0	24±3.07	24±2.7	27.6±3.6
Discrim. avoidance							
Learning (trials)	13.9±2.9	12.9±1.6	15.2±2.2	20.9±1*	13.3±1.1†	14.1±1.3†	10.7±0.9†
Reversal (trials)	9.6±1.1	9.8±1.3	9.1±.0.8	14.4±1.2*	11.85±1.1	10.4±0.8	9.7±0.9

[1] Learning index

[2] Probe

* $p<0.05$ individual comparison with young control

† $p<0.05$ individual comparison with age-matched controls

CHAPTER IV

BIOCHEMICAL ANALYSES

INTRODUCTION

Numerous studies have addressed the possibility of a link between oxidative stress and brain aging (Dubey, Forster, Lal, & Sohal, 1996; Sohal, Agarwal, Candas, Forster, & Lal, 1994; Sohal & Weindruch, 1996; R. Weindruch Sohal, 1997; R. Weindruch Walford, 1988). Ongoing studies in our laboratories have focused on the ability of senescence-initiated antioxidant supplementation to reverse or attenuate the accrual of oxidative damage, hypothesized to be a cause of age-related brain dysfunction. If the premise that brain dysfunction is related to the amount of oxidative damage is correct, then the ability of CoQ_{10}, Toc, or the combination of Toc+CoQ_{10}, to produce an improvement in cognitive or psychomotor function should be correlated with the ability to attenuate oxidative damage. This was evaluated in a series of as yet unpublished investigations by this investigator and Dr. Nathalie Sumien. In one set of investigations, whole brain mitochondria from the mice tested for cognitive and psychomotor functions in Chapters II and III were assayed for carbonyl content. Because mitochondria are a primary source of ROS as well as a primary target of these ROS, they could also be a primary target for the beneficial effects of those antioxidants. However, an additional study recently completed by Dr. Sumien indicated that beneficial effects of the

antioxidants, as well as their combination, may be region specific. The results of these studies are outlined in the following sections, and the outcomes are discussed in Chapter V in relation to the behavioral results presented in Chapters II and II.

Experiment 1: *Effect of CoQ_{10} intake during late life on mitochondrial protein oxidative damage*

Introduction

Experimental interventions like dietary supplementation with antioxidants have been used to lower the level of oxidative stress and potentially reduce age-associated oxidative damage. This experiment tested the effect of a large lipophilic antioxidant, CoQ_{10}, when its supplementation was initiated late in life. It has already been established that long-term CoQ_{10} supplementation failed to reduce age-associated protein oxidation in various tissues and had no effect on antioxidant enzymes systems, GSH/GSSG ratio, oxygen consumption, or ETC complex activity (Sohal et al., 2006). Lifelong supplementation with CoQ_{10} had no effect on various parameters of oxidative stress, however it is presently unclear whether or not senescence-initiated supplementation with CoQ10 may decrease oxidative damage. The present experiment was conducted in order to determine whether a relatively short term supplementation in old behaviorally characterized mice would reduce protein oxidative damage in mitochondria from whole brain and from skeletal muscles.

Animals and Treatment

Separate groups of male C57BL/6 mice were obtained from the National Institute on Aging at 3-4 months (n=24) and 17-18 months of age (n=31).The animals were

allowed to acclimate for a two-week period, following which they were randomly assigned to one of the three treatment groups. The mice were fed *ad libitum* either a control diet (base diet) or a diet supplemented with low (0.72 mg/g) or high (2.81 mg/g) concentration of CoQ_{10}. The daily dose of CoQ_{10} that the mouse received was either 105 mg/kg/day for a low dose or 368 mg/kg/day for a high dose, and this was based on the calculations of body weight and the average food intake of a mouse in one week. The mice were maintained on this diet for a period of approximately 10 weeks. After being assessed behaviorally (as described in chapter II), the mice were euthanized and mitochondria were prepared from brain and skeletal muscle to measure protein oxidation.

Isolation of Mitochondria

Mitochondria were isolated by differential centrifugation as per Sims for brain mitochondria and Trounce et al. for skeletal muscle mitochondria (Arcos, Sohal, Sun, Argus, & Brunch, 1968; Beattie, 1968; Sims, 1993; Trounce, Byrne, & Marzuki,1989). The mitochondrial pellets were resuspended in appropriate volume of buffer and stored at –80° C until further analyses.

Determination of protein carbonyl (CO) concentration

Protein oxidative damage was measured as protein carbonyls in brain and skeletal muscle mitochondria. Procedure to measure protein oxidation was a modified protocol of Levine et al., 1994 (Levine, Williams, Stadtman, & Shacter, 1994). Mitochondria were diluted in 5mM phosphate buffer (pH-7.5) resulting in 1mg protein/ml. Diluted mitochondria was added to 10 mM dinitophenylhydrazine (DNPH) in 2N HCl (samples) or 2N HCl (blank) in 1:5 ratio. The tubes were then incubated for 1 hr at room

temperature in the dark. The proteins precipitated on addition of trichloroacetic acid (TCA-10% final concentration) and finally the samples are centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the pellets were washed at least 3 times with 1ml ethanol/ ethyl acetate (1:1) and then dissolved in 650 µl of denaturing buffer (100µM of sodium phosphate buffer with 3% SDS at pH-6.8). The controls and samples were read at 360 nm while protein concentration was read using BCA protein detection system (Pierce, Rockford, IL) read at 530 nm on a spectrophotometer and nmols of carbonyl/mg of protein was calculated using the extinction coefficient of 22.0 $mmol^{-1}cm^{-1}$

Results and Discussion

There was an increase in CO concentration with age both in homogenates and mitochondria (Fig 1-left and right panel respectively), however, the increase in CO concentration in old mice brain mitochondria was only significantly different in brain mitochondria ($p > 0.022$) (Fig 1- right panel). Supplementation with a low and high CoQ_{10} diet in old mice indicated trends toward reduction of CO concentration when compared with their age-matched control but it was not statistically significant (all $ps > 0.056$). When CO concentration was measured in both skeletal muscle homogenates and mitochondria, there was no age-related increase in CO content and supplementation with both low and high doses of CoQ_{10} did not alter the CO content in the skeletal muscle (*all* $ps > 0.4$) (Fig 2). Single antioxidant supplementation with CoQ_{10} failed to decrease oxidative damage. Similarly supplementation with Toc alone also failed to reduce protein oxidative damage although there was an apparent increase in endogenous levels of Toc in the cerebral cortex (Sumien, Heinrich, Sohal, & Forster, 2004). These studies suggested

that single antioxidant supplementation failed to reverse or attenuate oxidative damage when initiated late in life.

Experiment II: *Effect of Toc, CoQ_{10} or CoQ_{10} +Toc intake during late life on mitochondrial protein oxidative damage.*

Introduction

Lifelong supplementation with CoQ_{10} or Toc alone did not delay age-related declines in cognitive and psychomotor functions and did not reduce oxidative damage. Senescence-initiated supplementation of these aformentionned antioxidants singly failed to decrease protein oxidative damage or improve age-associated decline in brain function. It is known that CoQ acts as an indirect antioxidant by regenerating α-tocopherol when the reduced form of CoQ reacts with α-tocopheroxyl radical (Kagan, Davis, Lin, & Zakeri, 1999; Maguire, V, Ackrell, Serbinova, & Packer, 1992). Short-term intake of CoQ_{10} in 12- and 24-month old rats increased cerebral cortex mitochondria levels of CoQ_{10} (Matthews, Yang, Browne, Baik, & Beal, 1998). Dietary supplementation with vitamin E + CoQ_{10} in rats resulted in significantly higher levels of CoQ_{10} in both tissue homogenates and mitochondria of liver and spleen. Similarly, rats fed a diet supplemented with CoQ had higher levels of Vitamin E in liver than those not supplemented with CoQ (Ibrahim, Bhagavan, Chopra, & Chow, 2000). A similar result was obtained by Lass and colleagues where an increased level of Vitamin E was reported in mice liver and skeletal muscle mitochondria following CoQ_{10} supplementation (A. Lass, Forster, & Sohal, 1999). Young mice supplemented with different doses of CoQ_{10} for 11 weeks had increased levels of CoQ_9 and CoQ_{10} in brain mitochondria. The same

study indicated that CoQ also affected the endogenous levels α-tocopherol in homogenates as well as mitochondria from different tissues (Kamzalov, Sumien, Forster, & Sohal, 2003). Mice supplemented with a diet containing Vitamin E alone, CoQ_{10} alone or combination of both resulted in a reduction in superoxide generation and this was inversely proportional to the level of α-tocopherol in the mitochondria (Lass, A., and Sohal,R.S., 2000). These studies indicate that there is a sparing/regeneration effect of CoQ_{10} on α-tocopherol thus the antioxidant action can be augmented by a diet supplemented with a combination (CoQ_{10} + α-tocopherol) than by a single antioxidant.

Animal and Treatment

Separate groups of male C57BL/6 were obtained from the National Institute on Aging at 3-4 months and 17-18 months of age (total of n=124) were fed *ad libitum* either a control diet (gamma-cyclodextrin in base diet), or the control diet supplemented with D- α-tocopheryl acetate (Toc) (200 mg/kg body wt/day) (Sigma Chemical Co.), or with CoQ_{10}, obtained from Tischon Corp. (148 mg/kg of CoQ_{10} daily intake) or with a combination of CoQ10 and D- α-tocopheryl acetate (200 mg/kg body wt/day + 148 mg/kg body wt/day). The mice were maintained on this diet for a period of 13-14 weeks. Following behavioral testing (described in detail in chapter III), the mice were euthanized and mitochondria were isolated from brain and skeletal muscle.

Determination of protein carbonyl (CO) concentration

Same protocol as experiment I was used to measure CO concentration.

Determination of thiobarbituric acid (TBARS) concentration

Lipid oxidative damage was measured as thiobarbituric acid reactive species in brain mitochondria. This procedure is modified from Ohkawa et al., 1979. Mitochondria (50 ul) was added to 300ul 20% acetic acid pH-3.5, 40 ul 8.1% SDS, 110 ul H2O and 300ul TBA. For standards, 6.07 uM of TMP was used (0, 10, 20, 30, 40 or 50 ul in 40% EtOH). After incubation at 95 ˚C for 60 min, the tubes were centrifuged at 3,000 rpm for 10 min. The standards and samples were read on a fluorometer (excitation:525 nm, Emission: 550 nm, k=5) (Ohkawa, Ohishi, & Yagi, 1979). The amount of thiobarbituric acid was calculated using the standard curve and the results were expressed as pmol TBARS/mg protein.

Results and Discussion

Oxidative damage was assessed by measuring protein oxidation (CO content) and lipid peroxidation (TBARS) in homogenates and mitochondria from brain and skeletal muscle of mice that were behaviorally characterized in chapter III.

Measurements in the brain indicated a trend toward a reduction in protein oxidation both in brain homogenates and mitochondria of old mice supplemented with antioxidants (Fig- 3 top panel). However, a one-way ANOVA with Treatment as a factor indicated no significant effect (all *ps*>0.35). Lipid oxidation as measured by pmol TBARS/mg protein in brain homogenates and mitochondria (Fig 3 bottom panel) indicated no reduction in lipid oxidation following antioxidant supplementation (all *ps*>0.74)

Determination of oxidative damage in skeletal muscle homogenate and mitochondria indicated that there was no reduction in protein oxidation following

antioxidant supplementation in skeletal muscle homogenate; however in mitochondria a trend in CO reduction was observed in the mice supplemented with vitamin E and VE+ CoQ10 (Fig 4 top panel). Analyses of variance indicated no significant main effect of Group (all *ps*>0.172). In skeletal muscle homogenate, there were trends toward reduction in TBARS with supplementation but no such trends were observed in mitochondria (all *ps*>0.41) (Fig 4 bottom panel).

Experiment III: *Effect of antioxidant supplementation alone and in combination on protein oxidative damage in different regions of the brain.*

Introduction

Our studies of mitochondrial protein oxidation were in progress when an additional study was initiated to assess the effects of Toc, CoQ_{10} and CoQ_{10} + Toc on protein oxidation within different regions of the brain. Previous studies suggested that the effect of age on oxidative stress was not uniform among different regions, when measured as protein oxidation or as shifts in the redox state of glutatione (Dubey et al., 1996; Rebrin, Forster, & Sohal, 2007). Thus, it was of interest to determine if the ability of antioxidants to attenuate oxidative stress was dependent on the region of the brain examined. The purpose of this study was to assess whether a combination of two synergistically-acting antioxidants, Toc and CoQ_{10}, was more effective in decreasing protein oxidative damage in various brain regions, than each antioxidant alone.

Animal and Treatment

Separate groups of male C57BL/6 mice 20 months of age, were assigned to one of the following treatment groups: vehicle (436 mg/kg/d γ-cyclodextrin (γ-CD)), Toc (250mg/kg/d α-tocopheryl acetate + vehicle), CoQ_{10} (109 mg/kg/d CoQ10 + vehicle) or Toc+CoQ_{10} (250mg/kg/d α-tocopheryl acetate + 109 mg/kg/d CoQ_{10} + vehicle). A group of 4-month-old mice was used as a young control group receiving the vehicle. The mice in these groups were gavaged daily with their respective treatments for a period of three weeks after which they were euthanized and the extent of protein oxidative damage,

measured as protein carbonyls (CO), was determined in homogenates of different brain regions.

Determination of protein carbonyl (CO) concentration

Same protocol as experiment I was used to measure CO concentration.

Results and Discussion

Determination of oxidative damage in various brain regions indicated that there was an age-related increase in CO in the cortex, hippocampus, cerebellum, and striatum of mice treated with the vehicle (Figures 5, 6, 7, and 8). One-way ANOVA with treatment Groups as a factor indicated there was a main effect of treatment group in cortex, cerebellum, hippocampus and striatum (all *ps* <0.049). Individual comparisons revealed that young control mice had significantly less CO than old control in cortex, hippocampus and striatum (all *ps*<0.049). Further, CO content was reduced in the striatum of old mice treated with Toc and Toc+CoQ_{10} when compared with age-matched controls (all *ps* <0.049). Toc +CoQ_{10} also significantly reduced CO content in the cortex of old mice when compared with age-matched control (p<0.036). In midbrain CoQ10 treatment indicated an increase in CO content that was significantly different when compared with their age-matched controls (*p*<0.008) (Fig 9). There was no effect of age or supplementation of antioxidants in brainstem (Fig 10).

In contrast to the effects of the antioxidants and their combinations on whole brain mitochondria, that suggested non significant trends toward attenuation of age-related increases in CO. These data also provide additional evidence to the published studies from our laboratory that the level of oxidative stress varies from region to region.

Further these data are also in agreement to the behavioral outcome where a cooperative effect of combination treatment was more beneficial in improving brain function than single antioxidant supplementation.

Fig 1: Effect of CoQ_{10} supplementation on carbonyl concentration in mice brain homogenates (left panel) and mitochondria (right panel). All values represent the mean ± SE of 3-6 samples.

* $p < 0.022$ when compared with young control

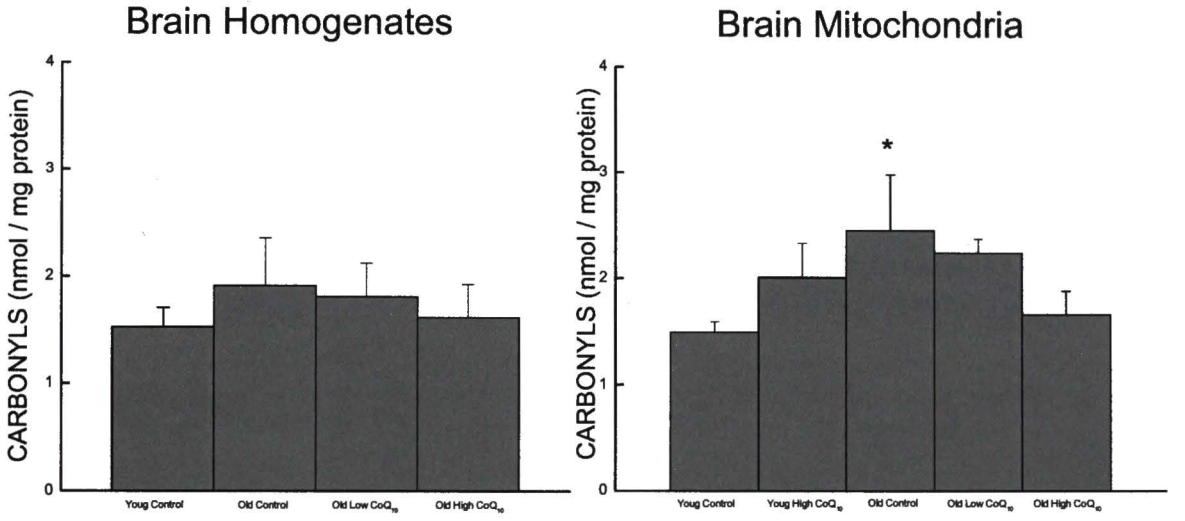
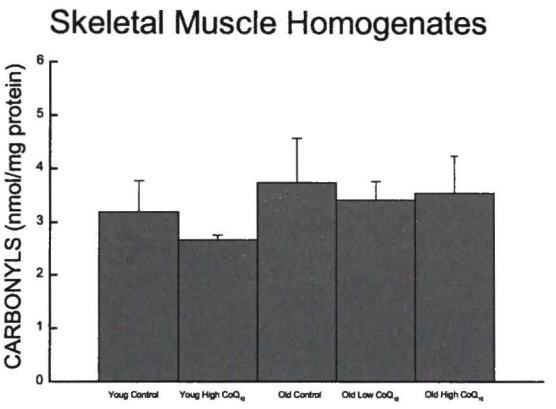

Brain Homogenates
CARBONYLS (nmol / mg protein)
0
1
2
3
4
Youg Control
Old Control
Old Low CoQ10
Old High CoQ10
Brain Mitochondria
CARBONYLS (nmol / mg protein)
0
1
2
3
4
*
Youg Control
Youg High CoQ10
Old Control
Old Low CoQ10
Old High CoQ10

Fig 2: Effect of CoQ_{10} supplementation on carbonyl concentration in mice skeletal muscle homogenates (left panel) and mitochondria (right panel). All values represent the mean ± SE of 3-6 samples.


Skeletal Muscle Homogenates
CARBONYLS (nmol/mg protein)
0
1
2
3
4
5
6
Youg Control
Youg High CoQ10
Old Control
Old Low CoQ10
Old High CoQ10

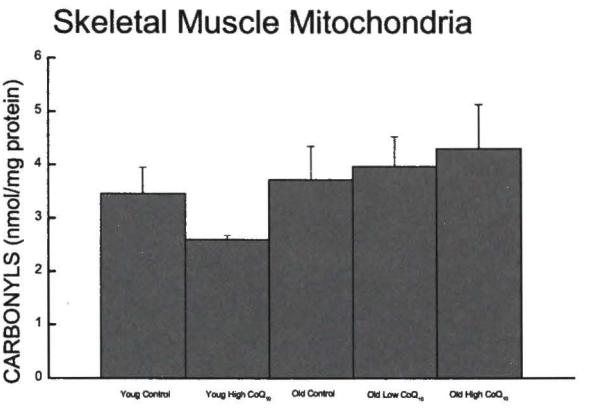

Skeletal Muscle Mitochondria
CARBONYLS (nmol/mg protein)
0
1
2
3
4
5
6
Youg Control
Youg High CoQ10
Old Control
Old Low CoQ10
Old High CoQ10

Fig 3: Effect of antioxidant supplementation on carbonyl concentration (top panel) and TBARS (bottom panel) in old mice brain homogenates (left panel) and mitochondria (right panel). All values represent the mean ± SE of 6-9 samples.

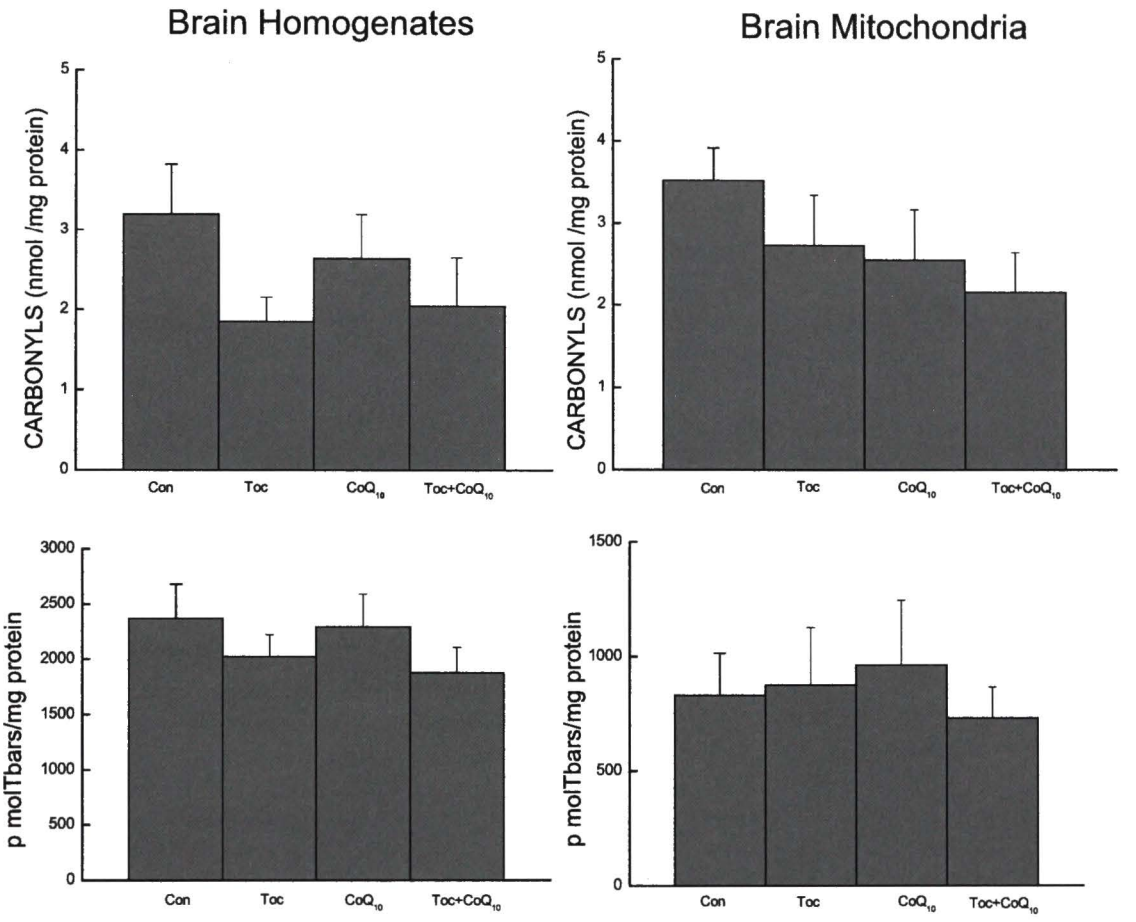

Brain Homogenates
Brain Mitochondria
CARBONYLS (nmol /mg protein)
p molTbars/mg protein
Con
Toc
CoQ10
Toc+CoQ10

Fig 4: Effect of antioxidant supplementation on carbonyl concentration (top panel) and TBARS (bottom panel) in old mice skeletal muscle homogenates (left panel) and mitochondria (right panel). All values represent the mean ± SE of 4-8 samples.

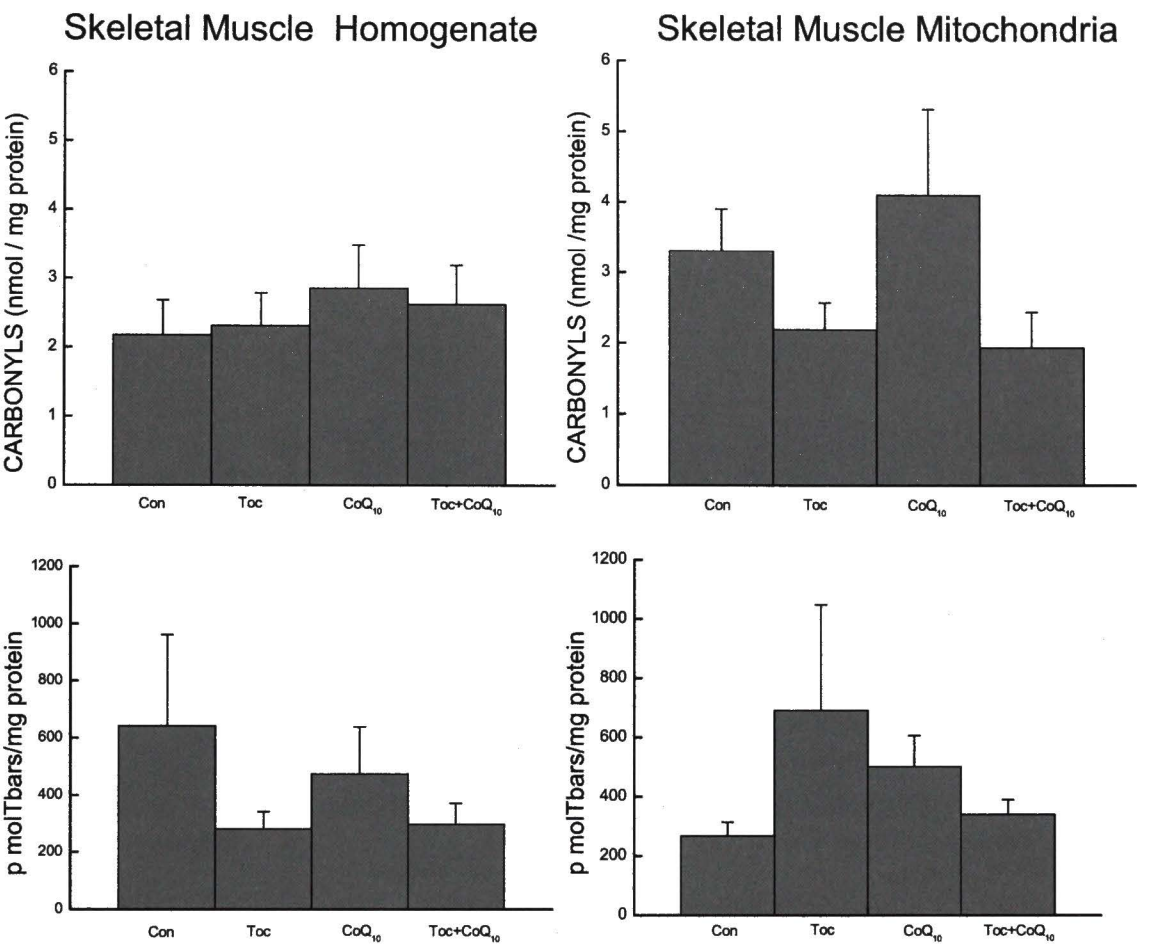

Skeletal Muscle Homogenate
Skeletal Muscle Mitochondria
CARBONYLS (nmol / mg protein)
CARBONYLS (nmol /mg protein)
p molTbars/mg protein
p molTbars/mg protein
Con
Toc
CoQ10
Toc+CoQ10

Fig 5: Effect of antioxidant supplementation on carbonyl concentration in mice cortex. All values represent the mean ± SE of 5-7 samples.

* $p < 0.049$ when compared with young control and + $p < 0.036$ when compared with age-matched control

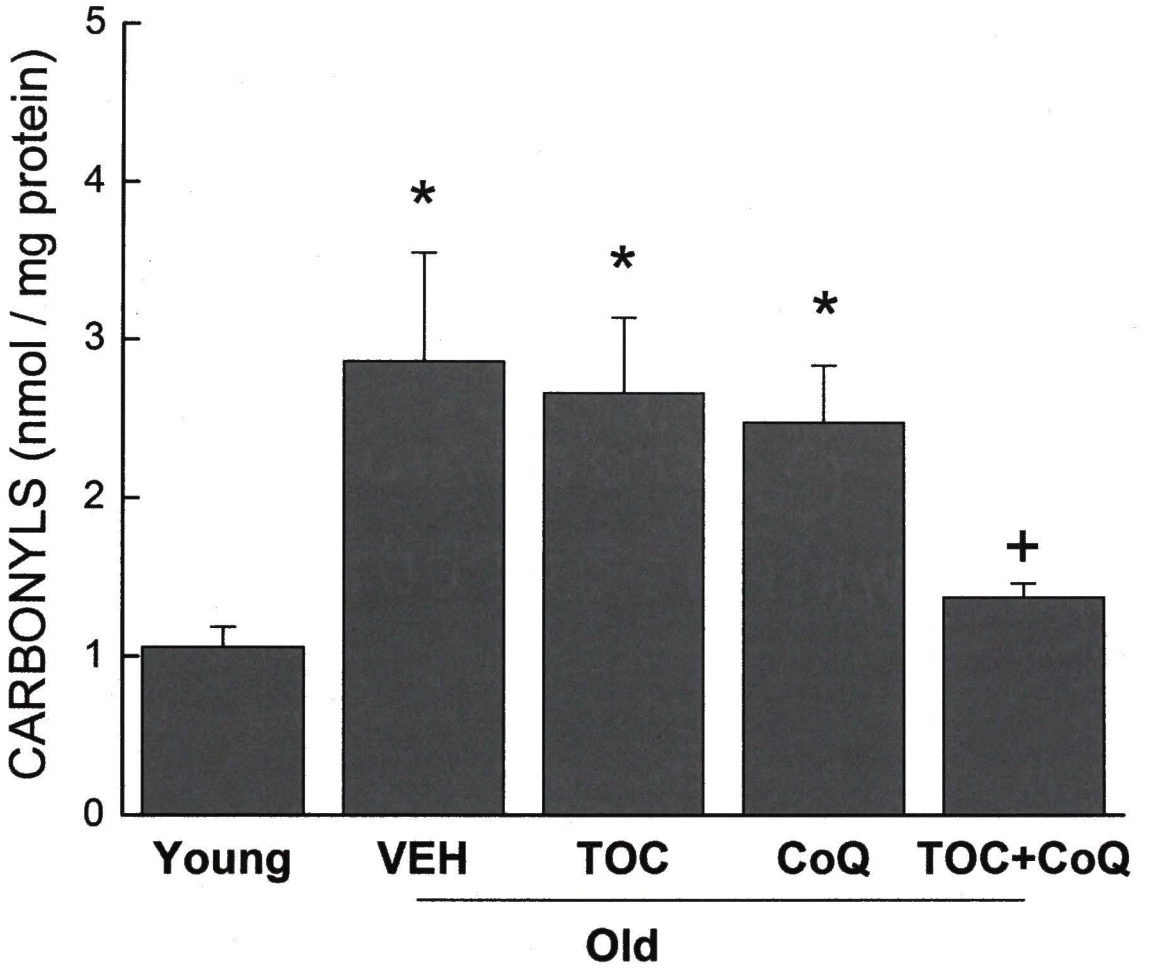

CARBONYLS (nmol / mg protein)
5
4
3
2
1
0
*
*
*
+
Young
VEH
TOC
CoQ
TOC+CoQ
Old

Fig 6: Effect of antioxidant supplementation on carbonyl concentration in mice hippocampus. All values represent the mean ± SE of 6-7 samples.

* $p < 0.02$ when compared with young control

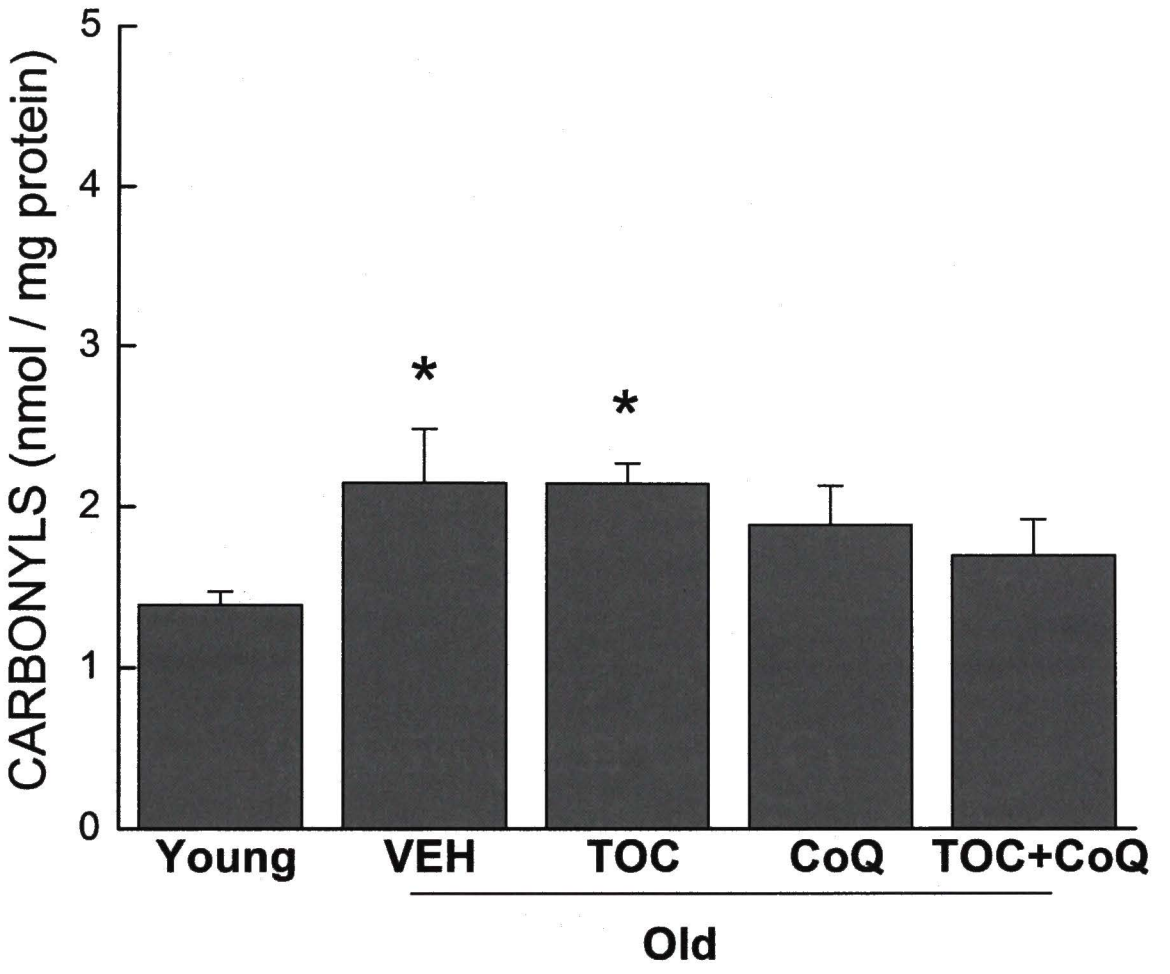

CARBONYLS (nmol / mg protein)
5
4
3
2
1
0
*
*
Young
VEH
TOC
CoQ
TOC+CoQ
Old

Fig 7: Effect of antioxidant supplementation on carbonyl concentration in mice cerebellum. All values represent the mean ± SE of 4-6 samples.

* $p < 0.03$ when compared with young control

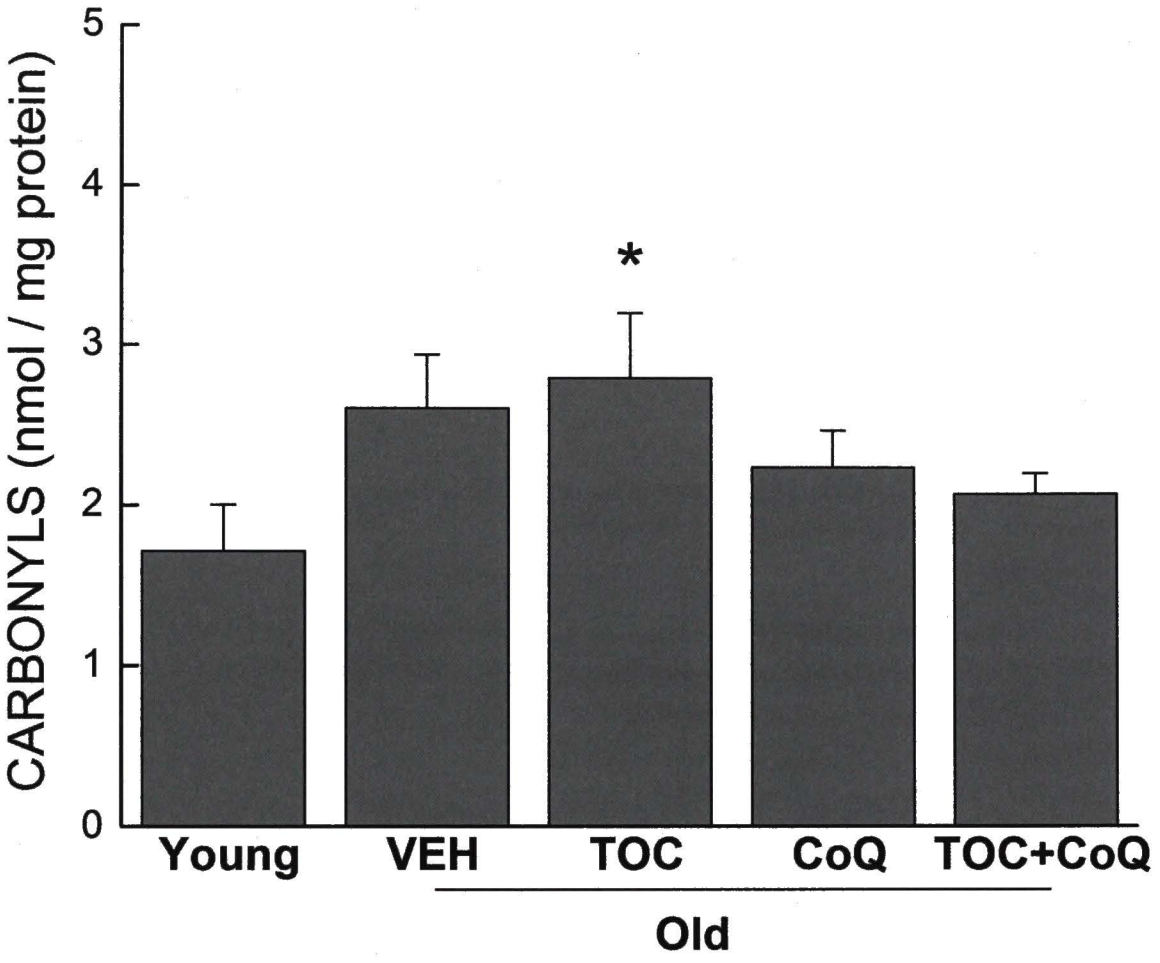

CARBONYLS (nmol / mg protein)
5
4
3
2
1
0
*
Young
VEH
TOC
CoQ
TOC+CoQ
Old

Fig 8: Effect of antioxidant supplementation on carbonyl concentration in mice striatum. All values represent the mean ± SE of 4-6 samples.

* $p < 0.007$ when compared with young control and + $p < 0.049$ when compared with age-matched control

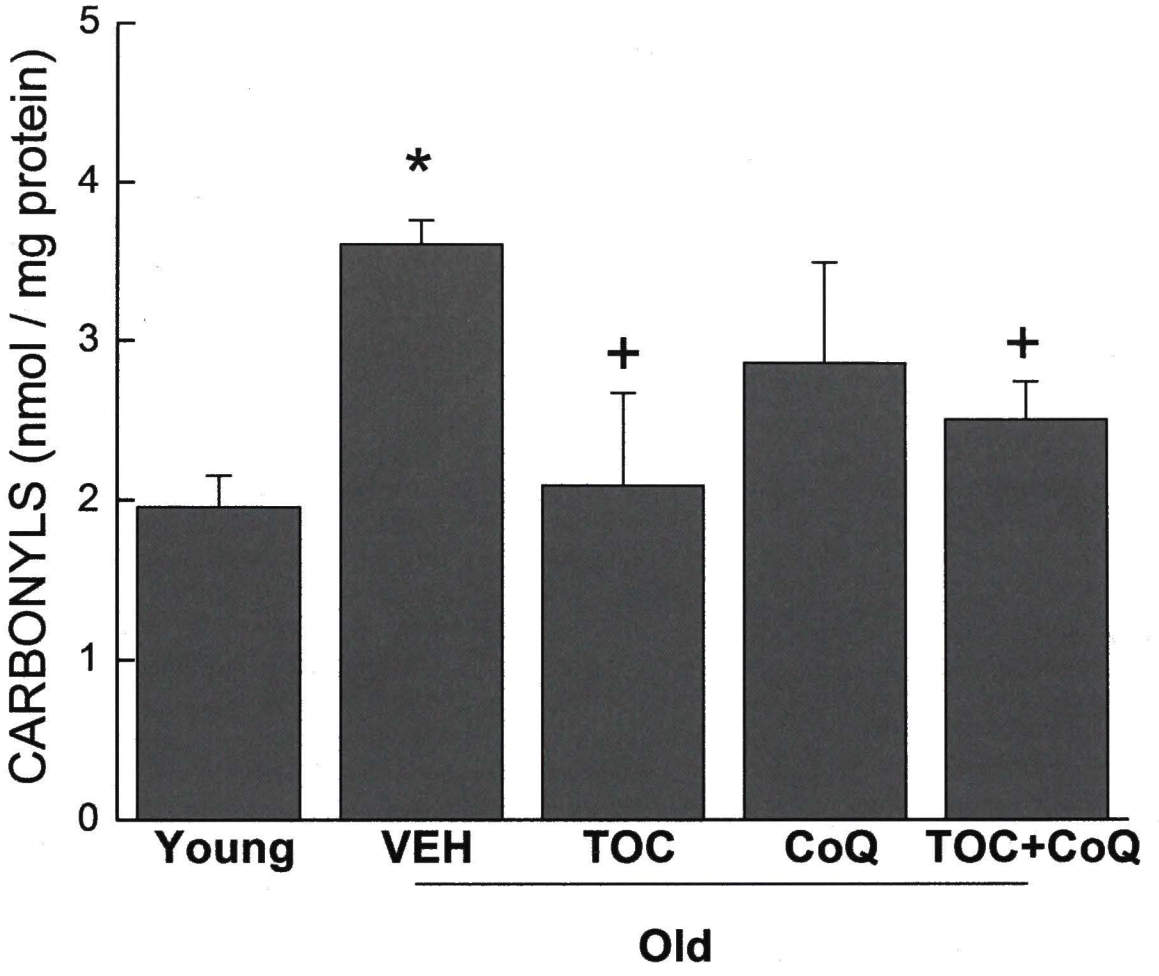

CARBONYLS (nmol / mg protein)
5
4
3
2
1
0
*
+
+
Young
VEH
TOC
CoQ
TOC+CoQ
Old

Fig 9: Effect of antioxidant supplementation on carbonyl concentration in mice midbrain. All values represent the mean ± SE of 4-6 samples.

* $p < 0.008$ when compared with young control and + $p < 0.008$ when compared with age-matched control

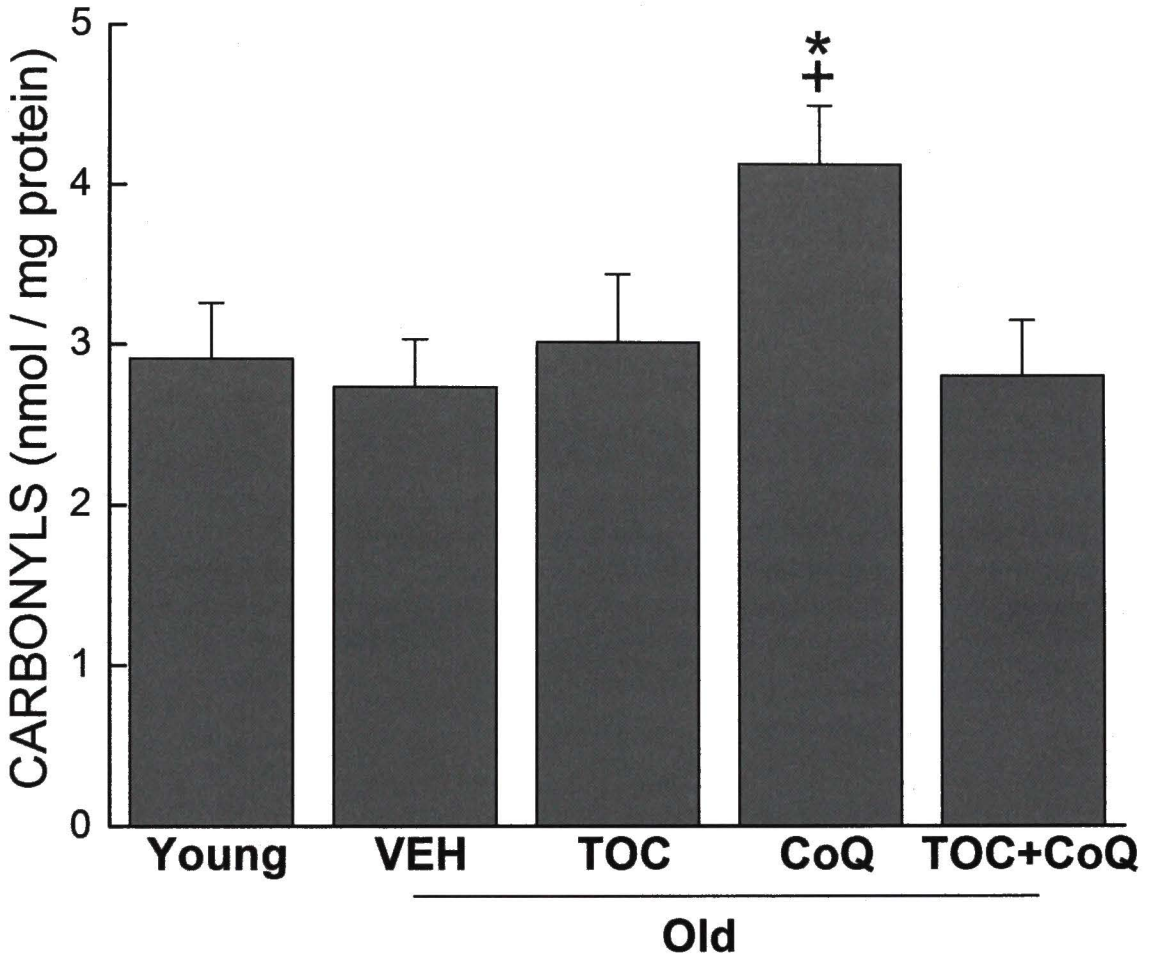
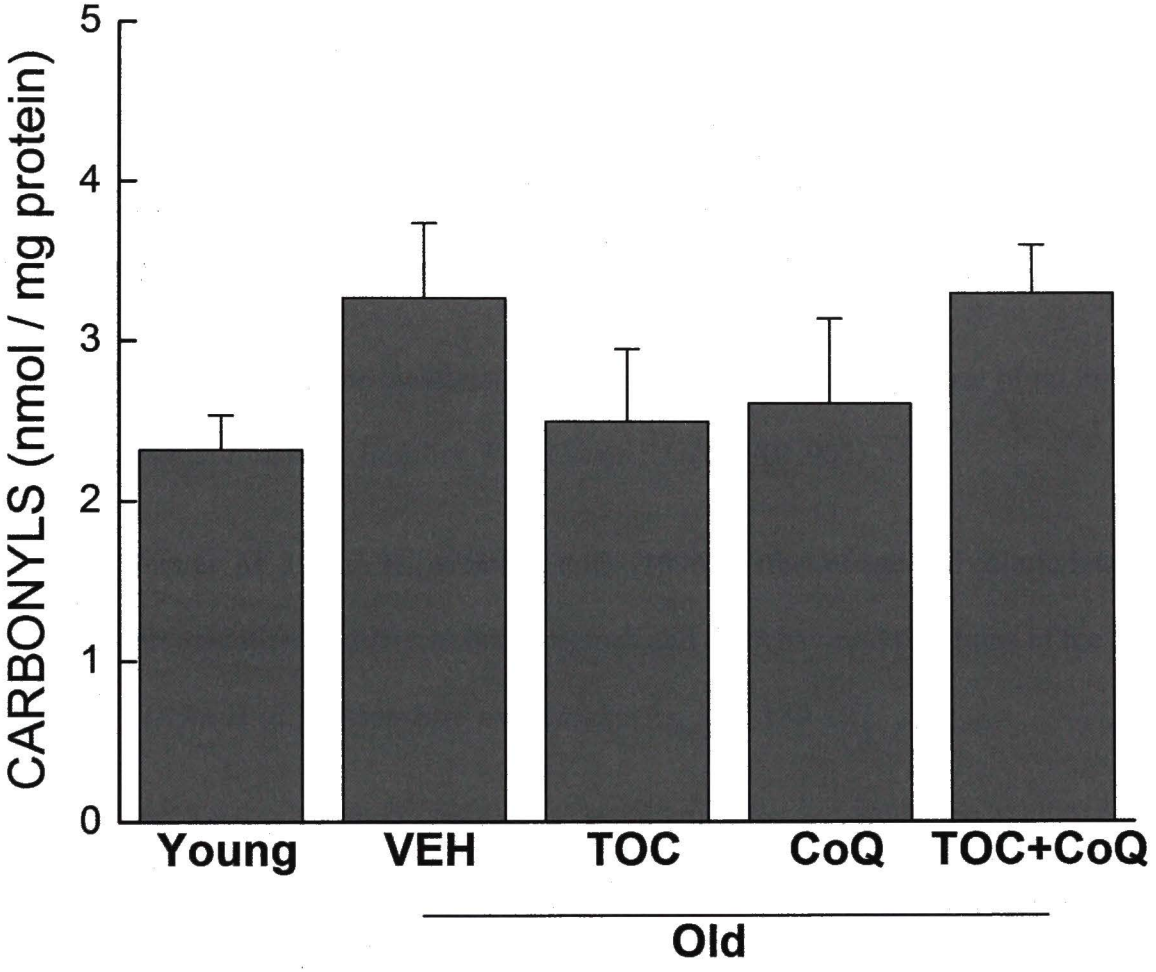

CARBONYLS (nmol / mg protein)
0
1
2
3
4
5
*
+
Young
VEH
TOC
CoQ
TOC+CoQ
Old

Fig 10: Effect of antioxidant supplementation on carbonyl concentration in mice brainstem. All values represent the mean ± SE of 4-6 samples.


CARBONYLS (nmol / mg protein)
5
4
3
2
1
0
Young
VEH
TOC
CoQ
TOC+CoQ
Old

Reference

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CHAPTER V

DISCUSSION

Aging is associated with mild to moderate loss of brain function that results in some loss of cognitive, psychomotor and sensorimotor capabilities. The mechanisms leading to this functional decline, although unclear, are thought to involve reversible alterations of numerous cellular and molecular substrates leading to functional loss, rather than a non-reversible loss of neurons. According to the oxidative stress hypothesis of aging, there is an inherent cellular imbalance involving the generation of reactive oxygen species in excess of cellular capacity for antioxidative defenses, leading to a time-progressive condition of "oxidative stress" that can result in an increase in oxidative modification of molecules, including lipids, proteins and DNA, that are crucial in cell function (Beckman & Ames, 1998; Sohal & Weindruch, 1996; Stadtman, 1992; Wickens, 2001). It has been established in studies of nonhuman primates, as well as many rodent species, that the functional loss and the cellular changes with aging are comparable to those occurring in aging humans (Gallagher & Rapp, 1997; Gower & Lamberty, 1993; Jucker & Ingram, 1997). Data from studies in rodents have indicated that there is a strong association between age-related increases in oxidative damage and age associated impairments in cognitive and psychomotor function (Carney et al., 1991; Forster et al., 1996; Kiray et al., 2006; Nicolle et al., 2001)

The link between oxidative damage and brain aging suggests that experimental interventions that can attenuate oxidative stress and produce a lowering of oxidative damage, or prevent its age-related accumulation, could prevent or ameliorate age-related brain dysfunction. Numerous studies of caloric restriction (CR) and antioxidant supplementation have addressed this possibility in animal models. In most studies, interventions have been implemented relatively early in life and maintained until old age. However, the current studies were based on the rationale that interventions could be effective even after significant brain aging has already occurred, in light of the potentially reversible nature of the brain changes responsible for functional impairments. It was hypothesized that amelioration of age-related cognitive and/or psychomotor impairments by antioxidant supplementation should be dependent upon their ability to decrease the steady-state amounts of oxidative damage. The results of the two research papers included in this document contribute substantially in evaluating the correctness of this hypothesis, especially when considered in light of new information regarding the ability of various antioxidant interventions to attenuate oxidative stress in the aging brain.

Tables 1 and 2 summarize short-term intervention studies conducted in our laboratory and their effects on oxidative stress/damage and behavioral function. Overall caloric restriction (CR) was able to reduce oxidative damage in different brain regions and also in skeletal muscles. This beneficial effect of CR at the cellular level was translated into improvements in psychomotor function (i.e. coordinating running and grip strength), however CR had marginal/or no effect on cognitive function.

Our first study (Chapter II) tested a widely used antioxidant, CoQ_{10}, and its potential benefits when supplemented in senescent mice. The results from the study confirmed the hypothesis that there was an age-related decline in cognitive as well as psychomotor function. These results also indicated that supplementation with CoQ_{10} when initiated in old mice did not improve motor or cognitive function, although there were some beneficial trends associated with CoQ_{10} supplementation on maximum spatial performance and acquisition learning index (LI) of the water maze test. However, these observed trends were independent of age, possibly reflecting improvement in ability of the mice in both age groups to locate the platform using non-spatial strategies. On the contrary, a deleterious effect of CoQ_{10} was evident in the young mice supplemented with a high dose of CoQ_{10} which performed more poorly in accurately locating the hidden platform in a probe trial. This deleterious effect could be explained by the possible pro-oxidant action of ubisemiquinone resulting in production of superoxide anion that may damage surrounding macromolecules (Linnane & Eastwood, 2006; Nohl, Gille, & Kozlov, 1998/10). The outcome from this study was in accordance with a previous study in our laboratory. Old mice supplemented with a high dose of CoQ_{10} (250 or 500 mg/kg/d) for a period of 14 weeks did not show improvement in performance on a discriminated avoidance task (McDonald, Sohal, & Forster, 2005). Furthermore, protein oxidation levels measured in homogenates and mitochondria from brains from the mice in Ch.1 indicated that there was no significant reduction in CO content with supplementation. From these two short-term CoQ10 intake studies, it can be concluded

that late life supplementation with CoQ_{10} alone was ineffective in modulating the cellular components that impact level of oxidative stress or behavioral function.

The ineffectiveness of CoQ_{10} in the recent studies could be attributed to the formulation of the diet. CoQ_{10}, being a large lipophilic molecule, is not very water miscible. A study by Bhagavan and colleagues indicated that the plasma concentrations of CoQ_{10} were higher in a solubilized (Q-Gel®) formulation than a powder form (Bhagavan & Chopra, 2007). Other studies from our laboratory have indicated an augmentation of CoQ in the brain following supplementation of Q-Gel® (Kwong et al., 2002; Lass, Forster, & Sohal, 1999).

Although a single antioxidant supplementation like CoQ_{10} proved to be not beneficial in aging mice, a recent study suggested that combining CoQ_{10} with another antioxidant α-tocopherol would have a higher antioxidant capacity in reducing the oxidative damage and enhance behavioral performance in old mice. The combination of two antioxidants, α-tocopherol and CoQ_{10}, assimilated in a diet improved the ability of mice to learn the discriminated avoidance task when compared to the performance of the animals fed a single antioxidant diet (indicated in Table 2)(McDonald et al., 2005). Similarly, treatment of old mice with bioenhanced CoQ from Tishcon corp. (120mg/kg/day) and α-tocopheryl acetate (275mg/kg/day) for a period of three weeks resulted in significant reduction in protein oxidation in the cortex of mice (unpublished data summarized in tables 1 and 2). Since it seems that the combination of Toc and CoQ was more effective in reducing oxidative damage in brain regions, a follow-up study was

designed to determine the effects of such a combination on psychomotor and cognitive function and oxidative damage in mitochondria in aged mice.

In the study discussed in chapter III, aged mice were supplemented with a base diet NIH-31 or the base diet containing α-tocophery acetatel (200 mg/kg body wt/day) or CoQ_{10}, obtained from Tischon Corp. (148 mg/kg body wt/d) or a diet containing a combination of CoQ10 and α-tocopheryl acetate. Both single antioxidant and combination of antioxidants supplementation significantly improved the performance of old mice in a discriminated avoidance task (Table 2). However, the combination of antioxidants had more impact in improving the performance on both sessions of the discriminated avoidance task. It can be concluded based on the aforementioned unpublished data that a cortical dependent task like discriminated avoidance can be improved by reducing protein oxidation in the cortex and this outcome was possibly due to a synergistic or additive effect of two antioxidants. Ubiquinol is known to regenerate α- tocopherol from the tocopheroxyl radical in the mitochondria and previous studies have shown that supplementation of CoQ_{10} can alter levels of α- tocopherol in the brain and that the level of α- tocopherol was found to be inversely proportional to the superoxide production in the mitochondria. (Lass & Sohal, 1998; Lass et al., 1999; Stoyanovsky, Osipov, Quinn, & V, 1995; V, Tyurina, & Witt, 1998). In the same study, the ability of old mice on a coordinated running task was significantly improved after supplementation with either α-tocopherol or with CoQ_{10}/α-tocopherol when compared to their age-matched controls (Table 1). This data was in contrary to the previous findings in our laboratory where neither α- tocopherol nor a combination diet of CoQ_{10}/α-tocopherol

benefited old mice in a coordinated running task; moreover, another study suggested that α- tocopherol supplementation when initiated late in life might deteriorate performance of old mice the same task. The coordinated running data obtained from the current study is puzzling but these contradictory data might be explained by a difference in the formulation of the diets between the studies. As mentioned in details in Chapter III, the soluble form of CoQ_{10} prepared in cyclodextrin was provided by Tischon corp.(Westbury, NY). In order to maintain consistency in all diets, equivalent amounts of cyclodextrin were added to the other diets, i.e. the control diet as well as the Toc diet. Cyclodextrin is widely used as an additive in the pharmaceutical industry to help solubilize highly lipophilic molecules (Pfitzner, Francz, & Biesalski, 2000; Szente, Szejtli, & Kis, 1998). The molecular structure of cyclodextrin forms a doughnut shaped inner cavity that allows large hydrophobic molecules like vitamin E or CoQ_{10} to be entrapped rendering them more water soluble and increasing their bioavailability (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). The data presented in chapter III represents a novel and unexpected finding in that improvement in coordinated running ability of old mice was evident after supplementation with an α-tocopherol diet containing the additive, cyclodextrin. Although previous investigators have reported an improvement in antioxidant activity of flavonols in the presence of cyclodextrin, an improvement in psychomotor performance has never been reported (Calabro et al., 2004).

The current findings, overall, are consistent with the hypothesis that oxidative stress/damage represents a significant cause of brain aging and associated cognitive and psychomotor impairments. However, the association between oxidative stress and aging

may be restricted to specific domains of neurobehavioral and brain function, and thus oxidation is clearly not a unitary cause of aging. Although a straightforward interpretation of the effects of antioxidants in the current studies would be that they afford a reduction in oxidative stress via conventional antioxidative action, it is also recognized that this action could occur as a result of modulating oxidative stress-associated signaling pathways. A schematic summary of the hypothesized sites of action for antioxidants is shown in Fig 1. Although there is no direct evidence between ROS, oxidative damage and aging, aforementioned literature discussed in chapter 1 indicates a strong correlation between the factors and a possible explaination to the aging/diseased state. As depicted in the fig.1 Toc in its dual role either scavenges free radicals therey reducing the steady-state level of ROS or acts on signal transduction molecules to prevent inflammation and oxidative damage. Tocopherol may also modulate the activity of PKC through PP_2A and prevent gene expression involved in cell adhesion (Aziz et al., 2002). CoQ in its antioxidant action is also known to scavenge free radicals and recycle Toc, thereby indirectly affecting cell function. Therefore combination of antioxidants has a synergistic or additive effect on complex physiological changes that contribute to aging and/or diseased state.

In conclusion, dietary supplementation of antioxidants improved psychomotor function relatively selectively. The improvements were limited to coordinated running ability after supplementation with either a Toc + γ-cyclodextrin diet or Toc+CoQ_{10}+ γ-cyclodextrin diet. Furthermore, a detrimental effect was observed with Toc only diet. These diets also reduced oxidative damage in the striatum and cortex, both involved in

coordinated learning. A combination of antioxidants Toc+CoQ_{10} with or without γ-cyclodextrin improved performance in the reversal phase of a discriminated avoidance task, a measure of cognitive inflexibility.

To date only caloric restriction and dietary supplementation of a combination of antioxidants had a significant impact in reversing age-related declines in specific psychomotor and cognitive function. Benefits of single antioxidant supplementation is limited and future studies should study the effect of single highly lipophilic antioxidants by formulating in a water miscible additive like cylcodextrin to increase the bioavailability in tissues.

Table 1: Attenuation of oxidative damage and amelioration of age-related psychomotor deficits

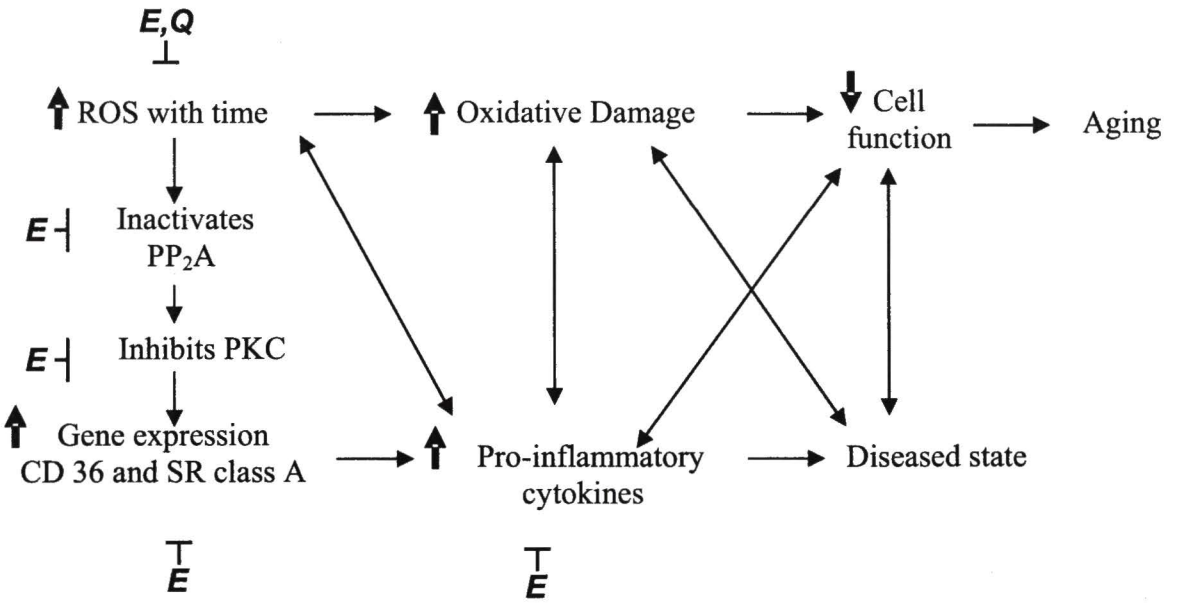
Late-life Intervention	Oxidative stress/damage						Psychomotor function		
	Cx	Hp	Str	Cb	Wb	Skm	Coordination (Rotorod)	Balance (Bridge)	Strength (Wire)
Caloric Restriction	$\downarrow^{1}$	$\downarrow^{1}$	$\downarrow^{1}$	$\downarrow^{1}$		$\leftrightarrow^{2}$	$\uparrow^{3}$	$\leftrightarrow^{4}$	$\uparrow^{3}$
Toc	$\leftrightarrow^{5}$					$\leftrightarrow^{6}$	$\downarrow^{5}$	$\leftrightarrow^{5}$	$\leftrightarrow^{5}$
Toc + γ-Cyclodextrin	$\leftrightarrow^{8}$	$\leftrightarrow^{8}$	$\downarrow^{8}$	$\leftrightarrow^{8}$	$\leftrightarrow\downarrow^{7}$	$\leftrightarrow\downarrow^{7}$	$\uparrow^{9}$	$\leftrightarrow^{9}$	$\leftrightarrow^{9}$
Coenzyme Q_{10}					$\leftrightarrow^{10}$	$\leftrightarrow^{10}$	$\leftrightarrow^{11}$	$\leftrightarrow^{11}$	$\leftrightarrow^{11}$
CoQ_{10} + γ-Cyclodextrin	$\leftrightarrow^{8}$	$\leftrightarrow^{8}$	$\leftrightarrow^{8}$	$\leftrightarrow^{8}$	$\leftrightarrow^{7}$	$\leftrightarrow^{7}$	$\leftrightarrow^{9}$	$\leftrightarrow^{9}$	$\leftrightarrow^{9}$
CoQ_{10} +Toc							$\leftrightarrow^{12}$	$\leftrightarrow^{12}$	$\leftrightarrow^{12}$
CoQ_{10} + Toc + γ-Cyclodextrin	$\downarrow^{8}$	$\leftrightarrow^{8}$	$\downarrow^{8}$	$\leftrightarrow^{8}$	$\leftrightarrow\downarrow^{7}$	$\leftrightarrow\downarrow^{7}$	$\uparrow^{9}$	$\leftrightarrow^{9}$	$\leftrightarrow^{9}$
ALA	$\leftrightarrow^{13}$	$\leftrightarrow^{13}$	$\leftrightarrow^{13}$	$\leftrightarrow^{13}$			$\leftrightarrow^{13}$	$\leftrightarrow^{13}$	$\leftrightarrow^{13}$

Table 2: Attenuation of oxidative damage and amelioration of age-related cognitive deficits

Late-life Intervention	Oxidative stress/damage					Cognitive function	
	Cx	Hp	Str	Cb	Wb	Spatial learning (Swim Maze)	Reversal Learning (Discriminated Avoidance)
Caloric Restriction	↓[1]	↓[1]	↓[1]	↓[1]			↔[13]
Toc	↔[5]					↔[5]	
Toc + γ-Cyclodextrin	↔[8]	↔[8]	↓[8]	↔[8]	↔↓[7]	↔[9]	↑[9]
Coenzyme Q_{10}					↔[10]	↔[11]	↔[11]
CoQ_{10} + γ-Cyclodextrin	↔[8]	↔[8]	↔[8]	↔[8]	↔[7]	↔[9]	↔[9]
CoQ_{10} +Toc							↑[12]
CoQ_{10} + Toc + γ-Cyclodextrin	↓[8]	↔[8]	↓[8]	↔[8]	↔↓[7]	↔[9]	↑[9]
ALA	↔[13]	↔[13]	↔[13]	↔[13]		↔[13]	↔[13]

1.Rebrin et al., 2007
2. Lass et al., 1998
3. Forster et al., 1999 (Neurobiol. Aging)
4. Shetty et al., 2004 unpublished
5. Sumien et al., 2004 (FRBM)
6. Sumien et al., 2003 (Exp. Gerontol)
7. Shetty et al., 2007 unpublished, reviewed in chapter IV (paper 2 mito.CO)
8. Sumien et al., unpublished, reviewed in chapter IV (gavage study)
9. Shetty et al., 2007., chapter III (paper 2 behavior)
10.Sumien et al., 2005, unpublished, reviewed in chapter IV (paper 1, mito. CO)
11.Shetty et al., 2007, chapter II (paper 1, behavior)
12. McDonald et al., 2004 (FRBM)
13. Shetty et al., 2002 unpublished
14. Forster et al., 1991 (Biomedical and Environmental Sciences)

Fig 1: Relationship between ROS, oxidative damage, inflammation, aging and diseased state. Abbreviations: E, α-tocopherol and Q,CoQ


E,Q
ROS with time
Oxidative Damage
Cell function
Aging
E
Inactivates PP2A
E
Inhibits PKC
Gene expression CD 36 and SR class A
Pro-inflammatory cytokines
Diseased state
E
E

Reference

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