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Aging is associated with a decline in psychomotor and cognitive function. Interventions such as exercise and antioxidants supplementation when investigated independently have been beneficial counteracting oxidative stress and improving brain function in both human and animals. A large number of health conscious individuals often combine exercise with vitamin supplementation, anticipating a synergistic effect maximizing their performance. While some studies reported additive effects, others have also indicated a potential for an antagonistic action of the antioxidants on the beneficial effects of exercise. To date, it has not been well established what the nature of the interaction between antioxidant supplementation and exercise is in terms of functional outcomes and whether age will influence the outcomes.

The purpose of this study was to determine if combination of antioxidant supplementation, and moderate exercise could ameliorate psychomotor and cognitive performance of young and old male mice

Using vitamins C and E and a treadmill-based forced exercise in young and old C57BL/6J mice, we explored the nature of that interaction on functional and biochemical outcomes. We examined the mice for spatial learning and memory, working memory and executive function, coordinated running performance, muscular reflexes, spontaneous locomotor activity, anxiety and muscle strength.

Our data suggested that the male mice exhibited age-associated declines in psychomotor and cognitive performance. Antioxidants supplementation worsened the cognitive flexibility of

old mice but improved the depression-like symptoms in young mice. Overall, exercise training reversed the age-related declines in reflexes and balance of old mice, and improved strength and associative learning of young mice only. Furthermore, combination of exercise and antioxidant improved reflexes, motor and cognitive performance, but additive or antagonistic effects of antioxidants on the beneficial effects of exercise were not observed.

Hence we can conclude that, combining antioxidants and exercise may not be provide any additional benefit in reversing age-related functional impairments.

ANTIOXIDANTS, EXERCISE, AND BRAIN FUNCTION

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ANTIOXIDANTS, EXERCISE, AND BRAIN FUNCTION

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INTRODUCTION

AGING

The human body goes through the inevitable multifactorial process of aging characterized by an increased vulnerability to disease (Mandavilli et al. 2002; Troen 2003; Harman 2003; Alexeyev et al. 2004; Peters 2006a). Recent advances in medical technology and aging research have facilitated human life expectancy (Olshansky et al. 2009). According to the United Nations (UN), our world population is undergoing a demographic shift that will result in much more older individuals in the coming years. The number of persons over age 60 will be reaching approximately 2 billion by 2050 (United Nations 2008). Although, there has been a dramatic increase in average human life expectancy, it is not necessarily associated with a better health span. Aged individuals are more susceptible to chronic diseases, including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases (Perry 2010; Vaupel 2010). This causes loss of autonomy, dependence, distress and high social costs for individuals and society, which will result in higher financial burden on our healthcare system. In addition, it is estimated that anti-aging enthusiasts will spend $191.7 billion by 2019 for treatments that mask the effects of aging (Market Research 2014). However, these interventions do not treat the cause of aging and to this day the key molecular mechanisms of aging remain undiscovered. While we may not have found the fountain of youth yet, aging research has made significant contributions in developing therapies. (HARMAN 1956; Yu 1996; Beckman and Ames 1998a; Shukitt-Hale et al. 1999; Ashok and Ali 1999; Kontush et al. 2001; Young and Woodside 2001; Biesalski 2002; Fang et al. 2002; Mandavilli et al. 2002; Grodstein et al. 2003; Harman 2003; Troen 2003; Weinert and Timiras 2003; Alexeyev et al. 2004; Sumien et al. 2004; Buffenstein 2005; Kramer et al. 2006; Peters 2006b; Glisky 2007; Olshansky et al. 2009; Fontana et al. 2010). These therapies help prevent or delay age-related diseases by understanding mechanisms underlying the effects of aging and thus help individuals to live longer

than before (Figure 1) (United Nations World population prospects. 2008). Some of the major theories of aging are discussed in the following paragraphs.

AGING AND OXIDATIVE STRESS

Many theories have been put forward to explain the phenomenon of aging and its impact on physiological and neurological function (Table 1) including: evolutionary theories (Weinert and Timiras 2003), the free radical theory (Harman 2003), the mitochondrial theory (Cadenas and Davies 2000), the immunologic theory (Franceschi et al. 2000) and the inflammation theory (Chung et al. 2001). One of the most robust and accepted Free radical theory of aging has been modofied to oxidative stress hypothesis of aging.

Oxidative Stress Hypothesis of Aging

Free radical theory

Free radicals are positively, negatively or neutraly charged atoms or group of atoms that has one or more unpaired electrons. Radicals are involved in a variety of normal biochemical reactions, and are generally unstable and very reactive (Fang et al. 2002). The free radical theory of aging was proposed by Denham Harman in 1956 (HARMAN 1956). According to the free radical theory of aging, cells continuously produce free radicals by normal metabolism and oxidation of organic compounds. The free radical causes damage to the cellular macromolecules (DNA, protein, and lipids), and accumulation of these changes over time is hypothesized to cause aging. This theory has been tested and supported by many research groups thus far (Cross et al. 1987; Sohal et al. 1994; Beckman and Ames 1998b; Ashok and Ali 1999; Biesalski 2002; Liochev 2013a).

Subsequent findings have led to a modified version of the free radical theory, the oxidative stress hypothesis of aging that proposes that reactive oxygen species (ROS) generated during mitochondrial respiration have the ability to damage cellular components, and are responsible for age-related functional loss (Sohal and Weindruch 1996; Beckman and Ames 1998a). ROS are highly reactive chemical species that can react with other molecules and become "reduced" in the process (Liochev 2013b). During aerobic respiration, electrons escape the electron transport chain and combine with surrounding oxygen to produce superoxide anion radical ($O_2^{\bullet -}$). In the living cell, superoxide anion radical is transformed by superoxide dismutase into hydrogen peroxide (H_2O_2) that can react with iron or copper to form the hydroxyl radical (·OH), which is extremely reactive and has the potential to oxidize and damage cellular components (Sohal and Weindruch 1996; Beckman and Ames 1998a). The prevention and repair of these damages are facilitated by detoxifying antioxidants such as vitamins E and C, as well as enzymatic antioxidants including superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase (Halliwell 1996; Cadenas and Davies 2000). Furthermore, other endogenous enzymes such as thioredoxin peroxidase and reductase, and glucose-6 phosphate dehydrogenase can protect cells from oxidative damage by maintaining a reducing environment (Beckman and Ames 1998a).

One of the key enzyme involved in the DNA repair is Poly (ADP-ribose) polymerase-1 (PARP), which utilizes nicotinamide adenine dinucleotide (NAD^+) as a substrate (Burkle et al. 2005). With aging PARP can be over-activated, which results in depletion of NAD^+ stores and subsequently causing brain dysfunction (Beneke and Burkle 2004). Earlier studies have indicated that vitamin E and vitamin C protects against apoptosis, DNA damage, and glutamate toxicity (Fischer-Nielsen et al. 1994; Zhang et al. 1997). A recent finding also suggests that Vitamin C protects neurons through inhibition of PARP-1 (Shah et al. 2015).

Mice deficient in α-tocopherol transfer protein (αTTP)-deficient mice with complete vitamin E deficiency lead to severe neurological disorders (Yokota et al. 2001a). Similarly, decreased vitamin E levels in the brain of transgenic phospholipid transfer deficient mice lead to deficits in affective and locomotor function (Desrumaux et al. 2005). Furthermore, decrease in endogenous vitamin C levels in the brain has been associated with impaired cognition, and elevated oxidative stress levels in aged mice (Dixit et al. 2015). During aging, production of ROS is stimulated in various tissues that lead to depletion of antioxidant stores and subsequently causing age-associated functional declines (Sohal et al. 1993; Cadenas and Davies 2000). Furthermore, studies involving knocking out SOD1 in flies or targeted inactivation of SOD1 in rodents provide a strong correlation between elevated free radical production and reduction of lifespan (Phillips et al. 1989; Finkel and Holbrook 2000; Elchuri et al. 2005). Also, caloric restriction without malnutrition reduces age-related mortality in rhesus monkey (Colman et al. 2014) and extend the lifespan of rodents, which is accompanied by reduced oxidative damage and improvement of other physiological parameters (Sohal and Weindruch 1996).

Interestingly, young naked-mole rats exhibit high oxidative stress which remains stable even at advanced ages (Lewis et al. 2013). Furthermore, in contrast to C57Bl6 mice, which show higher incidence of tumors, naked-mole rats are resistant to tumor and show minimal age-associated functional changes. Recent studies show that proteasomes of naked mole rats are more efficient in removal of soluble protein carbonyls and more resistant to formation of protein aggregates (Shringarpure and Davies 2002; Buffenstein 2005; Buffenstein 2008; Lewis et al. 2013).

Recently, researchers have discovered that ROS are implicated in cell signaling required for proper cell function. For example, physiological concentrations of ROS especially H_2O_2 is critical to cellular signaling and ontogeny (Beckman and Ames 1998a; Massaad and Klann 2011).

Recent studies demonstrated that suppression of ROS prevented long-term potentiation, a model of associative memory (Serrano and Klann 2004). Furthermore, recent reports suggest that increase in age-related functional losses does not always match the level of oxidative damage accumulated during aging (Muller et al. 2007; Perez et al. 2009a). Also, strategies aimed to augment intrinsic antioxidant defenses to counterbalance oxidative stress did not enhance the lifespan of mice (Mele et al. 2006; Jang et al. 2009; Perez et al. 2009b). Similarly, in most recent studies the under-expression of major antioxidant enzymes failed to shorten the lifespan in mice, except in Cu/ZnSOD-null mice (Sentman et al. 2006; Sentman et al. 2006; Perez et al. 2009b). As aforementioned ROS are now defined as physiologically vital molecules required for signal transduction, gene regulation, and redox regulation. . With these recent reports in minds, a revised version of the oxidative stress hypothesis has been advanced.

The Current State of Oxidative Stress Theory: The redox stress hypothesis of aging

The revised hypothesis proposes that with advancement in age antioxidant defenses weaken, and more oxidants are generated. This results in pro-oxidizing shift in the redox state of the cells resulting in over-oxidation of redox-sensitive protein thiols, and consequent disruption of the redox-regulated signaling mechanisms (Sohal and Orr 2012). In the cell, total GSH exists as free and bound to proteins. Glutathione reductase enzyme catalyzes the conversion of oxidized (GSSG) to the reduced form (GSH). Since, glutathione reductase enzyme is constitutively active and induced by oxidative stress, the amount of GSH is usually more as compared to the GSSG (Zitka et al. 2012). The GSH:GSSG ratio is commonly used as a measure of cellular redox (Carelli et al. 1997; Noctor and Foyer 1998; Locigno and Castronovo 2001; Townsend et al. 2003). Furthermore, the GSH:GSSG redox couple is also the main and most abundant contributor to redox potential (Chai et al. 1994; Maher 2006; Rebrin et al. 2011). With age, the glutathione redox state

becomes progressively more pro-oxidizing as indicated by the decline in GSH:GSSG ratio and associated age-related impairment in biological function (Rebrin et al. 2003; Rebrin and Sohal 2004; Maher 2006; Rebrin et al. 2011; Sohal and Forster 2014). Furthermore, recent studies have demonstrated that over-expression of glutamate cysteine ligase (GCL), which is the rate limiting enzyme in GSH production, helped to increase the lifespan of long-lived flies (Rebrin et al. 2011). Taken together, there are strong evidences to support the redox stress hypothesis. However, further studies will be needed to demonstrate conclusively the redox stress hypothesis and reveal the mechanisms that underlie the aging process.

Oxidative, Redox Stress and Brain Aging

It has been noted that aging is associated with an overall decline in physiological function, and impairments of sensory, motor and cognitive functions (Yan et al. 1998; Yan et al. 2000; Yan et al. 2000; Peters 2006a; Peters 2006a; Liu et al. 2013; Liu et al. 2013). The brain is particularly vulnerable to oxidative stress due to its high aerobic metabolism rate, abundance of substrates like iron and polyunsaturated fatty acids, relatively low antioxidant capacity, and non-replicating nature of its neuronal cells (Evans 1993) However, neurogenesis has been observed in adult brain regions in such as subventricular zone and the subgranular zone of the hippocampal dentate gyrus (Ma et al. 2009; Ming and Song 2011). Furthermore, the newly born neurons are believed to migrate to neighboring brain regions such as olfactory bulb and dentate gyrus, where they differentiate into specialized neurons or modulate memory function (Imayoshi et al. 2008).Alongside, an extensive body of evidence indicates that aging involves an increase in oxidative stress and results in oxidation of cellular protein, lipid and DNA (HARMAN 1956; Richter et al. 1988; Richter et al. 1988; Richter et al. 1988; Richter et al. 1988; Agarwal and Sohal 1994; Agarwal and Sohal 1994; Agarwal and Sohal 1994; Agarwal and Sohal 1994; Sumien et al.

2004; Sohal and Orr 2012; Sohal and Orr 2012). Several studies provided evidence that aged brains were more susceptible to protein oxidation, which may underlie age-related functional impairment (Carney et al. 1991). In a study by Forster et al., an increase in protein oxidation in the cerebral cortex, hindbrain, and cerebellum was significantly correlated with performance in the Morris water maze (MWM), walk initiation, and bride walking tasks, respectively (Forster et al. 1996). Furthermore, recent studies have revealed that effect of oxidative stress in the brain to be inconsistent (Wang and Michaelis 2010a). Research suggests that, while many brain neurons can cope with an increase in oxidative stress, some were selectively vulnerable (Serrano and Klann 2004). Due to this differential effect of oxidative stress, the affected neurons are usually first to exhibit functional decline during normal aging. For example, the first regions to undergo degenerative aging are the hippocampus and cerebral cortex which are responsible for age-related decline in cognition (Wang and Michaelis 2010b). Furthermore, declines in strength, balance, and coordination are also associated with oxidative damage in the basal ganglia and cerebellum (Forster et al. 1996).

Numerous studies have documented the important role of antioxidant GSH, as a defense against oxidative stress. GSH is widely distributed in most tissues, including the brain (Pileblad and Magnusson 1990). Interestingly, a decline in GSH levels is associated with aging and many age-related diseases (Currais and Maher 2013). Furthermore, depletion of GSH in the brain is associated with neuronal damage and changes in brain function associated with age (Ames et al. 1993; Shukitt-Hale et al. 1998; Shukitt-Hale et al. 1999; Currais and Maher 2013; LeVault et al. 2015). In a study, Shukitt-Hale et al. showed that intracerebroventricular injections of buthionine sulfoximine followed by dopamine induced reductions in brain GSH levels associated with motor and cognitive deficits in Fischer 344 (F344) rats (Shukitt-Hale et al. 1997; Shukitt-Hale et al.

1998). A recent study by LeVault et al. found that lower brain GSH levels are associated with poor water maze performance (LeVault et al. 2015). Taken together, these studies suggest alteration in the GSH levels might affect motor and cognitive function

There are abundant studies that have indicated the role of dysregulated redox state in pathophysiology of aging (Sohal and Orr 2012). Therefore, any interventions that would reduce oxidative or redox stress may prevent or delay age-related functional deficits. One such intervention is caloric restriction, which retards the aging process, delays the age-associated decline in physiological fitness, and extends the life span of organisms of diverse phylogenetic groups (Holehan and Merry 1986; Yu 1996; Fontana et al. 2010; Sohal and Forster 2014). However, the effect of CR does not seem to be universal and is not a practical options for many (Sohal and Forster 2014). Therefore, other interventions have been studied to alleviate the effects of aging; such as exercise and antioxidant supplementation. Their individual effects on brain function are introduced in the following paragraphs.

ANTIOXIDANT SUPPLEMENTATION AND AGING

An important intervention widely studied to reduce effects of aging is the intake of antioxidants. An antioxidant can be defined as "substances that delays or inhibits the oxidation of that substrate (Halliwell and Gutteridge 1995; Halliwell 1996). To demonstrate, recent studies indicate that antioxidant supplements are vital to maintain redox homeostasis in the living system (Young and Woodside 2001). Antioxidant supplements can act through various mechanisms such as 1) directly neutralize or decreasing ROS production, 2) prevent and repair ROS-related damage to biomolecules; and (3) elevating the endogenous antioxidant levels (Berger 2005).

Due to the lack of complete understanding of the mechanisms of aging, our endeavor to healthy aging has been challenging. To date most interventions aiming to reduce age-related

psychomotor and cognitive decline provide little to no benefit. However, aging research for several decades has revealed the importance of lifestyle modifications, which includes healthy eating habits such as diets rich in antioxidants, vitamins, and micro-nutrients. In this study we focus on dietary supplements rich in antioxidant such as vitamins E and vitamin C.

Vitamin C and Aging

Vitamin C (ascorbic acid) (Figure 1A) is a strong water-soluble antioxidant and provides first line of defense against free radicals generated during normal cellular metabolism, in whole blood and plasma (Fusco et al. 2007). Notably, humans, unlike other animals (except fruit bats, guinea pigs and primates) are unable to synthesize vitamin C, due to an inherited mutation in a key liver enzyme l-gulonolactone oxidase that is responsible for biosynthesis of ascorbic acid from glucose (Drouin et al. 2011) Therefore, humans are entirely dependent on exogenous sources to meet demands of the body (Levine 1986; Drouin et al. 2011). While, humans primarily obtain vitamin C from fruits and vegetables, chemically identical synthetic or food-derived vitamin C are available, which differ in bioavailability. Also, natural vitamin C is unstable when exposed to oxygen or certain minerals. An alternate source, L-ascorbyl-2-polyphosphate yields 25% ascorbic acid activity in a stable form, is commonly used in animal diets as a source of vitamin C (de Rodas et al. 1998).

In humans, lower doses of vitamin C intake (<60 mg/day) are associated with reduced risks of cancer, cardiovascular diseases and cataracts. While, higher doses of vitamin C intake (>60 mg/day) resulted in increases in high density lipoproteins, and decreases in low density lipoproteins oxidation, blood pressure and cardiovascular mortality (Bendich and Langseth 1995).

Vitamin C is involved in various metabolic reactions and is required for normal physiological functioning of brain among animals and humans (Gale et al. 1996; Bowman et al.

2009a; Tveden-Nyborg et al. 2012). Interestingly, the brain is minimally affected by vitamin C deficiency, due to presence of specialized sodium dependent vitamin C co-transporters (SVCT2) that actively transport vitamin C in exchange of sodium. First the ascorbate is transported from plasma into the cerebrospinal fluid and then from extracellular fluid into neurons (Qiu et al., 2007). This active transport builds a 4-fold vitamin C plasma gradient in rats (200-400 µmol/L in CSF, 60 µmol/L in plasma) and 3-fold in human (160 µmol/L in CSF, 40 to 60 µmol/L in plasma)(Costa et al. 2012).

Unlike neurons, astrocytes do not express SVCT2 transporter and instead rely on passive facilitated diffusion of oxidized ascorbate, dehydroascorbic acid (DHA) via Glucose transporters (GLUT-transporters). Vitamin C can thus cross the blood brain barrier (BBB) in its oxidized form using GLUT1 (Hotting and Roder 2013). It readily enters the brain and is retained in brain tissue in the form of ascorbic acid (Agus et al. 1997).

Furthermore, vitamin C gets differentially distributed among various regions of the brain with hippocampus and the frontal cortex containing the highest concentrations (Hansen et al. 2014). Furthermore, adequate levels of vitamin C improve survival and prevent cerebral hemorrhages in newborn transgenic mice deficient in vitamin C (Sotiriou et al. 2002; Harrison et al. 2010). Furthermore, insufficient vitamin C can reduce hippocampal volume and neuronal count. Also, vitamin C deficiency can worsen the cognitive performance of guinea pigs and elevate oxidation markers, i.e., protein carbonyls, in various tissues (Tveden-Nyborg et al. 2012).

Vitamin C supplementation improved the performance of old mice on elevated plus maze, a passive avoidance task, habituation-based task in a light-dark paradigm (de Angelis and Furlan 1995; Parle and Dhingra 2003; Shahidi et al. 2008). Furthermore, vitamin C reduced the dopamine depletion and oxidative damage induced by the 1-methyl-4-phenyl pyridinium ion (Wagner et al.

1986). In another study, vitamin C protected CA1 area of the hippocampus against β-amyloid-induced oxidative stress and cytokine release in rats (Rosales-Corral et al. 2003).

Recent studies in humans suggest that plasma levels of vitamin C reduced with aging and higher levels are found to be positively correlated with cognitive performance in elderly (Foy et al. 1999; Grodstein et al. 2003). Taken together, these studies suggest that vitamin C lowers oxidative stress and is a prime candidate for anti-aging therapeutics.

Vitamin E AND Aging

Vitamin E is a strong lipid-soluble antioxidant found in the hydrophobic interior of cell membranes and circulating lipoproteins (Williams 2004). There are 8 isoforms of vitamin E (alpha-, beta-, gamma-, and delta-tocopherols and tocotrienols), which have varying degrees of bioavailability and biological activity (Cook-Mills and McCary 2010; Usoro and Mousa 2010). The active forms of vitamin E are tocopherols, especially α-tocopherol (Figure 1B) and γ-tocopherol. Alpha-tocopherol is a slightly more potent antioxidant, which has been found to significantly increase median lifespan in C57BL/6 mice (Banks et al. 2010; Cook-Mills and McCary 2010), while γ-tocopherol has superior detoxifying properties against reactive nitrogen species (RNS) (Jiang et al. 2001).

The presence of special tocopherol transfer protein (TTP) allows greater uptake and retention of α-tocopherols as compared to other tissues, suggesting an important role of vitamin E in the brain (Yokota et al. 2001b). Furthermore, Sumien et al. observed concentration of α-tocopherol increased by 50-60% in the cerebral cortex of C57BL/6 mice after vitamin E supplementation (Sumien et al. 2004). Behavioral and biochemical studies suggest that vitamin E can detoxify free radicals, reverse oxidative stress, and improve learning and memory (Gilgun-

Sherki et al. 2001; Sung et al. 2004). While high plasma vitamin E levels in humans has been associated with better cognitive performance in elderly, vitamin E deficiency in humans and animals leads to neurological deficits (Gilgun-Sherki et al. 2001).

Our group has previously shown that short-term vitamin E supplementation failed to reverse age-related impairments of cognitive or motor function or to reduce markers of lipid peroxidation in the cerebral cortex (Sumien et al. 2004). In contrast, recent studies in our lab have demonstrated that short-term vitamin E supplementation improved motor function of old mice and reduced protein oxidation (Shetty et al. 2014). It should be noted that isoforms of vitamin E used (natural and artificial) in the two studies were different, and could explain the differences in outcomes. Vitamin E can be obtained from natural sources or synthesized artificially. Natural vitamin E is derived from vegetable oils, and due to its molecular structure has biological activity of 100% in most mammals (Brigelius-Flohe and Traber 1999) (Figure 2b). Synthetic vitamin E is generally derived from petroleum products, while semi-synthetic vitamin E is extracted from plant sources. The bioavailability of synthetic forms varies considerably depending on the isoforms (Yang et al. 2009).

It is well documented that both vitamins E and C are powerful antioxidants and ROS scavengers, which are critical for proper brain functioning (Chatterjee et al. 1975; Harrison and May 2009; La Fata et al. 2014). Furthermore, concurrent treatment with multiple antioxidants has been found to be more effective in ameliorating body defense against ROS. For example: vitamin C recycles oxidized form of vitamin E back to reduced form, and therefore further enhancing the vitamin E's antioxidant power, when taken along with vitamin C (Kontush et al. 2001). Also, recent studies suggest that decrease in vitamin E and C concentrations has been linked to the cellular and functional dysfunction in Alzheimer’s disease (AD) patients (Riviere et al. 1998). Besides,

vitamins E and C supplementation is capable of improving short-term memory, psychomotor performance, verbal memory, and overall mood in older individuals (Riviere et al. 1998; Lee et al. 2001; Arzi et al. 2004; Mazloom et al. 2013).

In contrast, a meta-analysis of 19 studies failed to show benefits of antioxidants intake on the risk of AD. However, smaller studies involving fewer participants showed beneficial effects. Similarly, Cache Country Study demonstrated that combination of vitamin E and vitamin C, but vitamin E, reduced risk of AD (Zandi et al. 2004). The effects of antioxidants in the clinical trials resulted in undesirable outcome. Several explanations have been proposed. For example; Lloret and colleagues suggested that the doses of antioxidants cannot be assumed to be universal and needs to be tailored according to antioxidant effect in each patient (Lloret et al. 2009). Other possibility is that antioxidants were administered at advanced ages, when neurons may have died or synapses may have been lost (Petersen et al. 2005). Previous studies also suggest that combination of antioxidants has more benefits as compared to monotherapy (Zandi et al. 2004). Furthermore, process of aging is complex and multifaceted. Therefore, lowering down of oxidative stress via antioxidants may not be sufficient to yield favorable benefits (Brewer 2010).

EXERCISE AND AGING

In the last two decades, research has recognized the importance of physical activity on the aging brain. Physical activity attenuates the memory and cognitive decline associated with normal aging and AD (Kramer et al. 2006; Ahlskog et al. 2011; Lautenschlager et al. 2012; Jak 2012).Furthermore, lifestyle modifications factors such as exercise are increasingly being recommended as an important strategy to counteract the deleterious effects of age on cognition (Warburton et al. 2006). It is believed that exercise-induced enhanced memory and long-term potentiation might be due to the increases the rate of neurogenesis within the dentate gyrus of the

hippocampus (van Praag et al. 1999; Brown et al. 2003; Pereira et al. 2007) and up-regulated expression of trophic factors such as brain-derived neurotrophic factor (BDNF) (Ma 2008; Costa et al. 2012). Interestingly, defective adult neurogenesis leads to impairment of working memory and learning (Ouchi et al. 2013). While, increased neurogenesis has been linked to better spatial memory performance of young rats in water maze (Uysal et al. 2005; Hotting and Roder 2013) and eight-arm radial maze (Anderson et al. 2000), Y-maze (Van der Borght et al. 2007), object recognition tasks (O'Callaghan et al. 2007).

In a 8-weeks study in rats, Leasure et al. found that voluntary wheel running involves running at high speed for short duration, and forced exercise involves a slower, and steady pace for longer periods of time. Also, anxiety levels were higher in forced exercise compared to voluntary exercise group (Leasure and Jones 2008a). Interestingly, both forms of exercise increased the number of surviving bromodeoxyuridine (BrdU)-positive cells in the dentate gyrus, but forced exercise ultimately produced more neurons than voluntary exercise (Leasure and Jones 2008a). Taken together, the differences mentioned above between the two forms of exercise might be responsible for their differential effects on brain and behavior.

Human and animal studies have suggested that, moderate exercise regimens causes adaptive changes in the redox-signaling pathway, and thus fortifies the endogenous antioxidant defense system against oxidative stress (Somani and Husain 1997; Somani and Husain 1997; Radak et al. 2001). Furthermore, moderate exercise prevents numerous chronic diseases (Blair et al. 1995; Myers et al. 2002; Navarro et al. 2004; Warburton et al. 2006; Ouchi et al. 2013) and increases life span and improved function of rats (Navarro et al. 2004) and humans (Myers et al. 2002).

In humans, a meta-analysis involving 2020 subjects in 30 trials, found that exercise training improved the fitness, physical and cognitive function of demented elderly persons (Heyn et al. 2004). In addition, regular physical activity improves cardiorespiratory fitness, motor function, cognitive speed, auditory, and visual attention of healthy older adults (Angevaren et al. 2008).

AGING AND INFLAMATION

Aging in healthy individuals is characterized by increase in circulating levels of pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α and C-reactive protein (CRP) (Pedersen et al. 2000; Bruunsgaard and Pedersen 2003). Recent studies demonstrate the inflammatory response is mediated by the ROS-mediated activation of toll-like receptors on a variety of immune cells (Gill et al. 2010). Therefore, interventions such as antioxidants and exercise are believed to prevent oxidative stress and possibly reduce age-associated inflammation.

A recent study by Chen et al. showed that Vitamin C inhibited lipopolysaccharide-induced, TNF-α, and IL-6 in macrophages cells (Chen et al. 2014b). Similarly, supplementation with antioxidants (multivitamin, vitamins E or C, beta carotene) lowered the serum levels of inflammatory markers such as (IL)-6 and CRP of elderly men and women (Colbert et al. 2004). Furthermore, recent studies indicate that acute bout or strenuous exercise increases inflammatory activity (Moldoveanu et al. 2001),while, regular moderate exercise in young and old individuals leads to a reduced level of inflammatory markers such as (TNF)-α and CRP (Mattusch et al. 2000; Tsukui et al. 2000). Physical activity may reduce inflammation by its effect on adipose tissue, which is the source of pro-inflammatory cytokines (Coppack 2001).

GOALS OF CURRENT RESEARCH

The overall goal of the proposed study is to evaluate the interactive nature of two-anti-aging interventions to protect against the negative functional outcomes of aging, and identify the underlying mechanism responsible for these improvements.

Data from recent studies have shown that antioxidant supplements and moderate exercise, when implemented independently appear to improve age-impaired cognitive and psychomotor performance by acting through similar mechanisms such as reduction of oxidative stress (Agus et al. 1997; Anderson et al. 2000; Young and Woodside 2001; Warburton et al. 2006; Angevaren et al. 2008; Leasure and Jones 2008b; Bowman et al. 2009b; Banks et al. 2010; Aguiar et al. 2011; Ahlskog et al. 2011). Therefore, it can be hypothesized that combining antioxidant with exercise training, a therapeutic approach employed by many health conscious individuals and recommended by healthcare professionals, will lead to an additive beneficial effect, (Devi and Kiran 2004; Jolitha et al. 2006; Cetin et al. 2010).

However, the nature of the interaction of the antioxidant supplementation and exercise has not been well established. Moreover, data from recent studies have revealed that antioxidant supplementation may interfere with exercise-induced cell signaling and consequently have an antagonistic action on the beneficial effects of exercise (Ristow et al. 2009; Paulsen et al. 2014). In a recent study, Paulsen and colleagues investigated the effects of vitamin E (235 mg/day) and C (1000 mg/day) supplementation for 11 weeks on endurance training adaptations in skeletal muscles of fifty-four young men and women (Paulsen et al. 2014). High dosages of vitamin E and C hampered the exercise-induced adaptive upregulation of mitochondrial biogenesis markers including cytochrome c oxidase subunit IV (COX4) and cytosolic peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC-1α). Furthermore, vitamin C prevented exercise-

induced adaptive increase in markers of mitochondrial biogenesis such as cytochrome C and antioxidant enzymes (Gomez-Cabrera et al. 2008). Ristow and colleagues found that 4 weeks of vitamin C (1000 mg/day) and vitamin E (400 IU/day) supplementation antioxidant (1000 mg/day) supplements may prevent the health-promoting effects of exercise on type 2 diabetes mellitus, by blocking the exercise-induced molecular regulators of insulin sensitivity and endogenous antioxidant defense (Ristow et al. 2009). In another study involving six mildly hypertensive older men (71+/-2 years), oral antioxidants (vitamins C and E, and alpha-lipoic acid) blunted the beneficial effects of exercise on hypertension and exercise-induced vasodilation (Wray et al. 2009).

In addition to these studies, few studies failed to report any negative interaction between antioxidants and exercise interventions. Moderate exercise combined with dietary vitamins C and E, reduced ROS and blood glucose levels in the kidney and lens of streptozotocin-induced diabetic rats (Kutlu et al. 2005). Supplementation with vitamins C and E reversed the exhaustive exercise-induced oxidative damage and improved cognitive function young male mice (Rosa et al. 2007). In another study involving 11 men and 11 women ultramarathon runners, 6 weeks of supplementation with vitamins E (300 mg) and C (1000 mg) could prevent exercise-induced lipid peroxidation, but had no effect on inflammatory markers (Mastaloudis et al. 2004). Similarly, antioxidant supplementation had no effect on the exercise-induced lipid peroxidation and muscular damage induced by exhaustive such as strenuous bicycle or marathon exercise training (Kaikkonen et al. 1998; Yfanti et al. 2010)

Taken together, studies mentioned above indicate that there is a definite interaction between exercise and antioxidant supplementation, however the nature of such interaction seems dependent on context such as type of exercise, or age of subject/animals. Furthermore, only a few

studies have looked at this interaction in relation to brain aging. The present study was designed to characterize the nature of the interaction between antioxidant supplementation and exercise on brain dysfunction associated with aging. The goals of the current study were 1) to determine whether antioxidant intake and exercise training led to beneficial improvements in young and old C57BL/6J mice 2) to determine whether the combination of antioxidant and exercise yield an additive beneficial effect; and lastly 3) to determine whether the beneficial outcomes are age-dependent.

The overall hypothesis of this study is that combination of antioxidants with exercise will maximally reverse age-related psychomotor and cognitive dysfunction.

Figure 1: Life expectancy at birth by region, 1960-2045

Each value represents life expectancy in years across Asia, Africa, Developed countries, Latin America and Caribbean, and the World. (United Nations World population prospects, 2008)

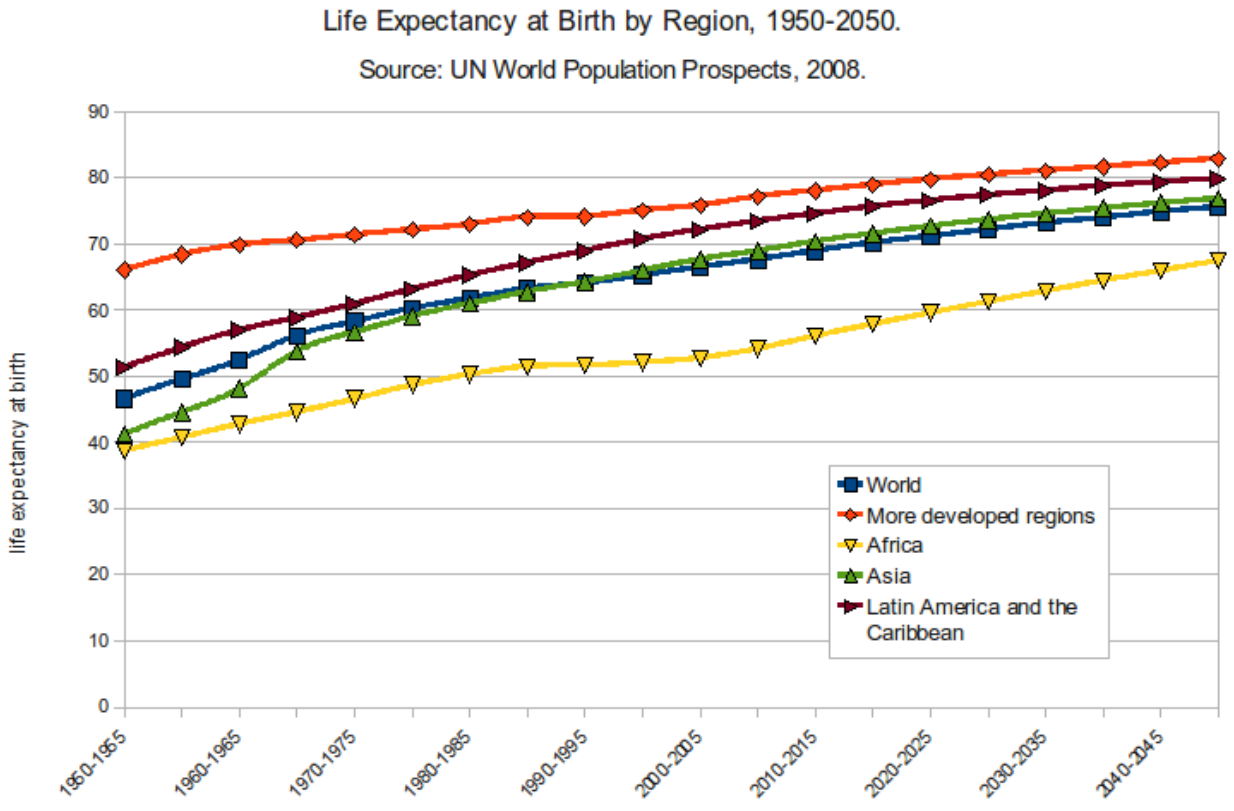
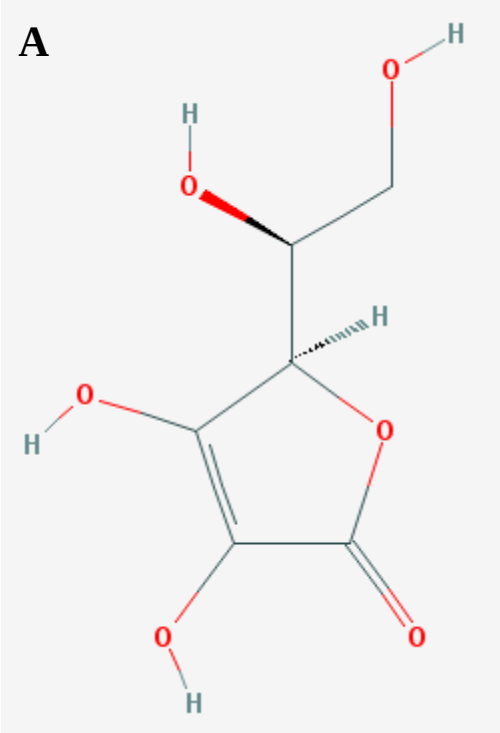
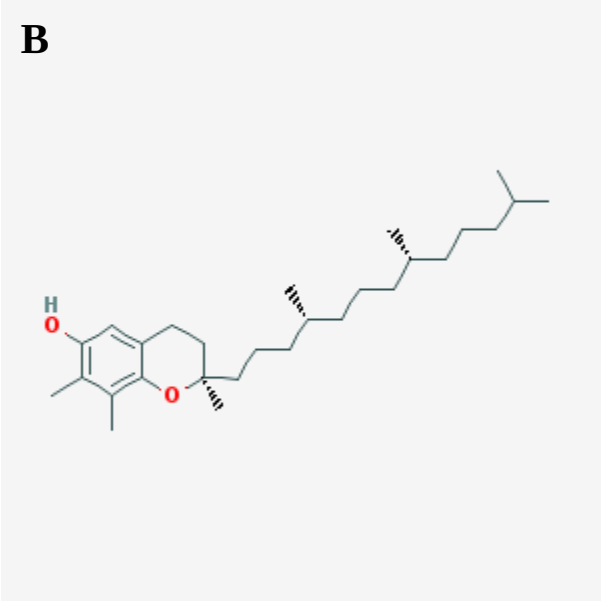

Life Expectancy at Birth by Region, 1950-2050.
Source: UN World Population Prospects, 2008.
life expectancy at birth
90
80
70
60
50
40
30
20
10
0
1950-1955
1960-1965
1970-1975
1980-1985
1990-1995
2000-2005
2010-2015
2020-2025
2030-2035
2040-2045
World
More developed regions
Africa
Asia
Latin America and the Caribbean

Figure 2: Structures of (A) vitamin C and (B and C) vitamin E (Yamamoto et al. 2001) (http://pubchem.ncbi.nlm.nih.gov)

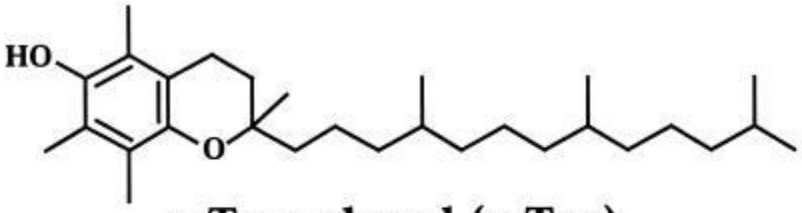
A



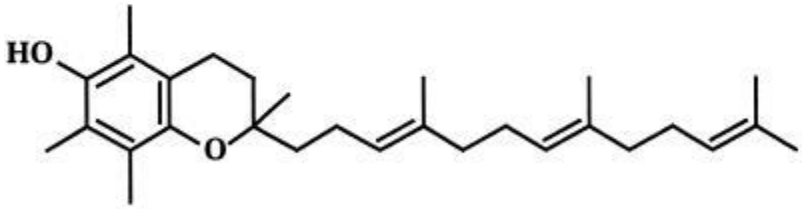
B



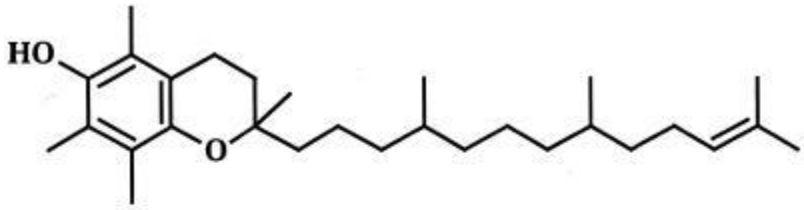
C



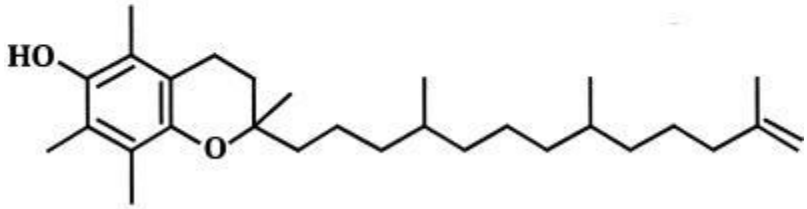
α-Tocopherol (α-Toc)



α-Tocotrienol



α-Tocomonoenol



Marine-derived tocopherol (MDT)

Table 1: Classification and brief description of main theories of aging (Weinert and Timiras 2003)

Biological Level/Theory	Description
Evolutionary	
Mutation accumulation	Mutations that affect health at older ages are not selected against.
Disposable soma	Somatic cells are maintained only to ensure continued reproductive success; after reproduction, soma becomes disposable.
Antagonistic pleiotropy	Genes beneficial at younger age become deleterious at older ages.
Molecular	
Gene regulation	Aging is caused by changes in the expression of genes regulating both development and aging.
Codon restriction	Fidelity/accuracy of mRNA translation is impaired due to inability to decode codons in mRNA.
Error catastrophe	Decline in fidelity of gene expression with aging results in increased fraction of abnormal proteins.
Somatic mutation	Molecular damage accumulates, primarily to DNA/genetic material.
Dysdifferentiation	Gradual accumulation of random molecular damage impairs regulation of gene expression.
Cellular	
Cellular senescence-Telomere theory	Phenotypes of aging are caused by an increase in frequency of senescent cells. Senescence may result from telomere loss (replicative senescence) or cell stress (cellular senescence).
Free radical	Oxidative metabolism produces highly reactive free radicals that subsequently damage lipids, protein and DNA.
Wear-and-tear	Accumulation of normal injury.
Apoptosis	Programmed cell death from genetic events or genome crisis.
System	
Neuroendocrine	Alterations in neuroendocrine control of homeostasis results in aging-related physiological changes.
Immunologic	Decline of immune function with aging results in decreased incidence of infectious diseases but increased incidence of autoimmunity.
Rate-of-living	Assumes a fixed amount of metabolic potential for every living organism (live fast, die young)

CHAPTER 2

MATERIALS AND METHODS

ANIMALS

All animal protocols were approved by the Institutional Animal Care and Use Committee at UNT Health Science Center at Fort Worth. A total of 82 4 months old and 83 20 months old male C57BL/6J mice were obtained from the National Institute on Aging, aged rodent colony and subsequently maintained in the UNT Health Science Center vivarium. The mice were housed in groups of 3 or 4 in standard polycarbonate cages (28 × 17 × 12.5 cm) with corncob bedding and ad libitum access to food and water, and were maintained at ambient temperature (23 ± 1 °C), under a 12-h light/dark cycle starting at 0600 hours. The mice were weighed weekly, and survival was monitored throughout the study. The mice were acclimated for a 1-week period, following which they were randomly assigned to one of the diets (control or antioxidant supplemented), and one of the exercise treatment (sedentary or exercised).

TREATMENT

Diets. The mice were fed either a control diet (LabDiet® R&M 5LG6 4F, cat #: 5S84) or the control diet supplemented with vitamins E and C (modified 5LG6 with 1.65 mg/g diet of ascorbate and 1.12 IU/g diet of α-tocopheryl acetate, cat#: 5SH0). The daily doses of ascorbic acid and α-tocopherol received were ~164 mg/kg/day and ~242 IU/kg/day respectively, and this was based on the calculations of body weight and the average food intake of a mouse.

Exercise. The mice also followed a sedentary regimen or a moderate force exercise regimen. The moderate exercise regimen, using treadmills (AccuPacer Treadmill; Omnitech Electronics Inc., Columbus, OH, USA), was introduced progressively over a 12-day period. The training was gradually incremented in time and speed to reach a maximal exercise of 1 h (6, 8, 10, and 12 m/min for 5 min each, and then at 14 m/min for 40 min). The training protocol used was a modification of previously published exercise protocols (Chaudhari et al. 2014). Forced exercise

was implemented via transient 0.29 mA electric foot shock to the feet. Each exercise mouse was paired with a sedentary mouse which received the same number of shock for each training day.

The mice were randomly assigned to one of four experimental groups: (1) sedentary fed the control diet (SedCon), (2) sedentary fed the vitamins E and C supplemented diet (SedEC), (3) forced exercise fed the control diet (ExCon), (4) forced exercise fed the vitamins E and C supplemented diet (ExEC).

The mice were on their respective treatments for 8 weeks prior to and throughout behavioral assessments for a total of 16 weeks. At 6 months of age for young and at 22 months for adult, the mice received a series of behavioral tests for cognitive, affective or psychomotor functions. Subsequently, mice were euthanized and brain regions prepared for measurement of glutathione (GSH, GSSG), and catalase activity

FOOD INTAKE

Food intake was monitored twice during the course of the study: the week prior to the start of the behavioral testing and the week after the completion of the behavioral testing. The food intake measurements were done over 5 consecutive days, at the same time each day to control for any diurnal variations. The amount of food consumed was normalized to the number of mice in each cage to estimate individual food intake.

FUNCTIONAL ASSESSMENT OF MICE

The behavioral studies evaluated the effect of treatments on brain functions. The behavior test battery has been well established and extensively in our laboratory in different studies in a variety of mouse strains and under various experimental conditions. Each of the protocols

addresses cognitive, sensory or psychomotor functions that can readily be studied in human subjects. Administration of the complete test battery requires approximately 8 weeks. The behavioral assessments were performed one at a time in the following order; elevated plus maze, spontaneous activity, coordination, walk initiation, alley turn, wire suspension, bridge walking, , water maze, discriminative avaoidance and tail suspension test Unlike previous studies, cognitive tests were focused on hippocampal function (Morris Water Maze: spatial discrimination) with visible platform being tested after to avoid confounding effect on the water maze, and cortical function (active avoidance: T-maze), allowing us to study two different domains. The complete battery of behavioral tests to be used in this project has been detailed in several publications from our group (Table 1; (Sumien et al. 2006; Shetty et al. 2013a; Chaudhari et al. 2014).

Locomotor activity

Each mouse was placed in a clear acrylic chamber (40.5× 40.5 ×30.5 cm) that was surrounded by a metal frame lined with photocells (Model 71-CPPX, Omnitech Electronics). The test chamber was enclosed in a dimly lit (31.8 ± 1.5 lux), sound-attenuating chamber (Model 71-ECC, Omnitech Electronics) equipped with a fan that provided background noise (64dB). 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 software (Fusion v. 5.3) to yield variables describing horizontal, vertical, and spatial components of spontaneous activity in the apparatus.

Motor learning

The apparatus was a motor-driven rotorod (Omnitech Electronics, Model # AIO411RRT525M) that consisted of a 3-cm diameter nylon cylinder mounted horizontally at a height of 35 cm above a padded surface. On each trial, the mouse was placed on the cylinder,

which then began rotating with increasing speed (0.5 revolutions per minute) until the animal fell to the padded surface. The 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, which represented each mouse's maximum stable level of performance. The average latency to fall was averaged for each session and on the last session for each mouse.

Simple reflexes

Walk initiation. Each mouse was placed on a flat surface and the latency to move one body length was recorded.

Alley turning. Each mouse was placed head first in a 3.5-cm wide, 14-cm long, dead-end alley and the latency to reverse direction was recorded. Each test lasted a maximum of 60 s and was administered once over four consecutive daily sessions, and the data are presented as the averaged latency from the 4 sessions.

Wire suspension

This test was administered twice each day over four consecutive days. For each trial, the mouse was allowed to grip a horizontal wire with its front paws when suspended 27 cm above a padded surface. Each trial lasted a maximum of 60 s and the latency to tread (reflexive grasping of the wire with the hind legs) and the latency to fall were recorded and averaged.

Bridge walking

This test was administered three times each day over four consecutive days. For each trial, the mouse was placed in the middle of bridge suspended between two platforms 50 cm above a

padded surface. Each day the bridge was different in diameter (small or large) and shape (round or square), providing four levels of difficulty. Each trial lasted a maximum of 60 s, and the latency to fall from the bridge was measured and averaged over the four days.

Morris water maze (MWM)

Spatial learning and memory were measured using an MWM test modified from described previously (55). On a given trial, the mouse was allowed to swim in a tank filled with opacified water and maintained at 24 ± 1 °C. The mice were able to escape the water by means of a hidden platform (1.5 cm below the surface of the water). A computerized tracking system recorded various measures such as path length and swimming speed (Any-maze; Stoelting Co., Wood Dale, IL, USA).

The test consisted of four phases: (1) *pre-training phase*: the tank was covered by a black curtain to hide surrounding visual cues. The mice learned the components of swimming and climbing onto a platform using a straight alley that had a platform at one end. The mice were allowed to swim until they reached the platform or a maximum of 60 s had elapsed. The mice received two sessions (Friday and Monday) consisting of five trials with an inter-trial interval of 5 min; (2) *acquisition phase*: the black curtain was removed and the mice were tested for their ability to locate a hidden platform using spatial cues around the room. Each daily session consisted of five trials, at 2-min intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. The mice were allowed to swim until they reached the platform or a maximum of 90 s had elapsed. Testing was conducted over nine sessions (Tuesday-Friday and Monday–Friday). On sessions 2, 4, 5, 7, and 9, a probe trial was conducted as the fifth trial during 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; (3) *retention phase*: one 60-s probe trial session was conducted 1 week after the ninth session of the previous phase (only the first 30-s were used for analyses to match the other probe trials); (4) *visible platform phase*: the mice were given a total of eight sessions (2/day separated by 2 h), each consisting of five trials with a 10-min inter-trial interval. The platform was identified by a triangular flag (with a large red dot in the middle of it for contrast) that was raised above the surface of the water. On each trial the mouse had to swim to the platform from a different starting point and the platform was moved to a different location before each trial. Thus, the mouse had to learn to associate the location of the flag with location of the platform. Only the final session (session 8) was considered for analyses of visual function, rather than learning.

Path length (distance taken to reach the platform) over sessions was used as the primary measure of performance. The path-independent swim speed was calculated by dividing distance by the latency to reach the platform. On probe trial, spatial bias for the platform location was evaluated in terms of the percentage of time spent within a 40-cm diameter annulus surrounding the platform location.

Discriminated avoidance

A T-maze constructed of acrylic (black for the sides and clear for the top) was utilized for the discriminated avoidance task. The maze was divided into three compartments: a start box (10 × 6.3 × 6 cm), a stem (17.5 × 6.3 × 6 cm), and two goal arms (14.5 × 6.3 × 6 cm), each separated by clear acrylic doors. The maze rested on a grid floor wired to deliver 0.69-mA scrambled shock to the feet.

The test consisted of three sessions separated by 1 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 (information trial), the mouse received shock in the first arm entered (preference arm) 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 of which the last two must be within 5 s. The second and third sessions of avoidance training were reversals such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous session. Their ability to learn the discrimination component of the task was considered inversely proportional to the number of trials required to reach the avoidance criterion aforementioned (4 out 5 correct turns regardless of the time taken).

Tail suspension test

The test procedure described by Steru et al. was used to test depressive behavior with slight modifications (Steru et al. 1985). The method is based on the observation that a mouse suspended by its tail shows alternate period of activity and immobility (activity reflects efforts to correct the posture, and immobility reflects despair and lack of hope). Mice were suspended from their tails at a height of 20 cm using a piece of adhesive tape wrapped around the tail 2 cm from the tip. A mouse was considered to be immobile when it was suspended passively and completely motionless

and mice that climbed their tails were excluded from the data. Recordings were carried out for a total period of 6 min and treatment groups were compared with age-matched control groups.

Preparation of tissue homogenates

Mice were euthanized by cervical dislocation and each mouse brain was dissected into six regions: cerebral cortex, cerebellum, midbrain, brain stem, striatum, and hippocampus, and frozen at -80°C until further assessments. Each brain region was homogenized in phosphate buffer (50 mM potassium phosphate, pH 7.0, containing 1mM EDTA) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 0.1% Triton-X 100 or as recommended by the kit manufacturer. Homogenates were spun at 10,000 x g for 15 min; the pellets were discarded and the supernatant was used. The blood was collected by making a small cut at the base of the tail using scalpel blade. Plasma was separated from blood by spinning at 2500g for 10 minutes.

Oxidative stress markers

Catalase activity was determined by utilizing Catalase Assay Kit (Cayman Chemical Company Ann Arbor, MI 48108) that utilizes the peroxidatic function of catalase for the determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes its color from colorless to purple (540 nm). Catalase activity was calculated using the formula:

$$\text{Formaldehyde conc. of the sample } (\mu M) = \left(\frac{\text{Sample Absorbance} - (\text{y-intercept})}{\text{Slope}} \right) \times \frac{0.017 \text{ ml}}{0.02 \text{ ml}}$$

$$\text{Catalase activity} = \frac{\mu M \text{ of sample}}{20} \times \text{sample dilution} = \text{nmol/min/ml}$$

GSH/GSSG ratio in the brain of mice was determined by utilizing Glutathione Assay Kit by Cayman. This assay kit utilizes a carefully optimized enzymatic recycling method for the quantification of glutathione. Glutathione reductase (GR) reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). GSH is easily oxidized to the disulfide dimer GSSG. The sulfhydryl group of GSH reacts with DTNB (5,5′-dithiobis-2-nitrobenzoic acid, Ellman's reagent) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB) that absorbs at 414 nm. The rate of TNB production is directly proportional to the concentration of glutathione in the sample. The measurement of the absorbance of TNB at 414 nm provides an accurate estimation of glutathione in the sample.

Protein Assay

All protein concentrations were determined using the bicinchoninic acid (BCA) Protein Assay Kit from Pierce Biotechnology (Rockford, IL) with bovine serum albumin as a standard. The BCA Protein Assay is based on the mechanism that proteins convert Cu2+ to Cu1+ in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu1+) by bicinchoninic acid. In the first step, also known as biuret reaction the protein chelates the copper to form a light blue complex in alkaline medium. In the second step BCA reacts with the reduced (cuprous) cation that was formed in step one to form a purple colored reaction product which can be detected spectrophotometrically at 562 nm.

Plasma inflammatory markers

Inflammatory markers, IL-6, and TNF-α, were measured using a V-Plex Proinflammatory Panel 1 (mouse) Kit (K15048D-2; Meso Scale Discovery) according to manufacturer's instructions. The manufacturer's range of detection for each analyte is 0.40–3140 pg/ml.

Proinflammatory markers were measured using special sandwich immunoassays that utilized electrochemiluminescence as the basis for detection. The sample was added to pre-coated plates with capture antibodies along with solution containing detection antibodies conjugated to electrochemiluminescent labels, which emitted light when voltage was applied through electrodes. Lastly, a detection buffer was added to provide an optimum environment and the light signal was quantified by using charge-coupled device.

STATISTICAL ANALYSIS

Functional performance of the mice on the behavioral tests and biochemical measures were subjected to two-way analyses of variance (ANOVA), with Age and Treatment as between-groups factors. Planned individual comparisons between different age and treatment groups were performed using single degree-of-freedom F tests involving the error term from the overall ANOVA. Weights, water maze, and coordinated running data were subjected to three-way analyses with Session or Time as the repeated measure. The α-level was set at 0.05 for all analyses. The software used for the analyses was Systat 13 (Systat Software Inc., San Jose, CA, USA

Table: Summary of behavioral test battery

Test description	Capacity or deficit measured	Primary anatomical target of test
Sensory/Reflexive		
Alley turning	Reflexive, motor planning	Cortical orienting circuitry; dorsomedial frontal cortical
(-) geotaxis	Reflexive, arousal	
Locomotor/affective		
Initiation latency	Arousal	Various; striatum; brainstem/midbrain arousal circuitry; limbic reward circuitry
Horizontal	Ambulation, arousal	
Vertical	Rearing, arousal	
Spatial analysis	Anxiety/fear level	Limbic system
Tail Suspension Test	Depression	
Psychomotor		
Wire suspension	Muscle strength, coordination	Skeletal muscle, reflex circuitry
Grip strength	Fore-hindlimb muscle strength	Skeletal muscle
Bridge walking	Balance, coordination	Cerebellum,striatum
Rotorod	Coordination, motor learning	Striatum, cerebellum, premotor cortex
Swim speed	Coordination, motor learning	Striatum, premotor cortex
Cognitive		
Spatial water maze	Visual/spatial discrimination, explicit reference learning/memory; executive function/planning	Hippocampus, frontal cortex
Discrimination reversal	Associative learning (conditioned fear), cognitive flexibility	Frontal cortex, limbic system

CHAPTER 3

RESULTS

Body weight and food intake

Weekly body weights were recorded throughout the duration of the study and are presented in Fig.1A. Overall, the old mice weighed more than the young mice throughout the study. The sedentary young mice gained ~5-7% weight in 16 weeks, while the exercised young mice exhibited a stable weight throughout the study. In the old group, weights were decreased as a function of weeks, even more so in the treated mice (~9-13%) than in the SedCon ones (~2%). A repeated measure ANOVA revealed a significant interaction of Weeks, Age, and Treatment ($P < 0.001$) supporting the aforementioned observations. Survivorship was also followed: 1 young mouse died (ExEC group), and 7 old mice (1 from ExCon, and 2 from each of the other groups) died at various times throughout the study.

Food intake was measured before and after behavioral testing and is presented in Fig. 1B. Overall, the old mice ate more than the young ones at both time points. Effects of treatments were only observed after behavioral testing, with the 20-months SedEC group eating 26% less food than the age-matched SedCon group. Separate ANOVAs on the before and after food intake revealed significant main effects of Age ($P < 0.05$), but no effect of Treatment or interaction between Age and Treatment (All Ps > 0.219).

Locomotor activity

Three measures were selected in the analyses of spontaneous activity: distance travelled (Figure 2A), vertical activity also called rearing (Figure 2B), and time spent in the center of the testing apparatus (Figure 2C). The old mice travelled 19% less distance and reared 30% less than their young counterparts. These observations were supported by main effects of Age for each measure (all Ps < 0.018). While a main effect of Age was also found for center time ($P < 0.05$), there was no difference within the control groups (P=0.384) and the main effect was driven by age

differences within the SedEC and ExEC groups (all Ps <0.031). Treatments did not affect the distance of traveled of either young or old, as supported by a lack of main effect of Treatment or an interaction with Age (all Ps > 0.385). Treatments, ExCon and ExEC, decreased rearing and increased time spent in the center in young mice, but had no effect in the old ones. Two-way ANOVAs revealed main effects of Treatment for both measures (all Ps < 0.001), and an interaction between Age and Treatment for vertical activity (P <0.001).

Motor learning

The effects of age and treatment on the ability of the mice to learn a motor task are presented in Figure 3. Across sessions, the mice learned to remain on the rotating rod, as evidenced by increased latencies to fall and supported by a main effect of Session as repeated measure (P< 0.001). Overall, the older mice had shorter latencies than the young mice, and treatments had minor effects. In young mice, only the ExEC group exhibited higher latencies than the old ones, while in the old mice, ExCon and ExEC both had higher latencies. A two-way ANOVA indicated a significant effect of Age and Treatment, but no interaction between Age and Treatment (all Ps > 0.05), and no interaction of Age or Treatment with Sessions (all Ps > 0.072).

Reflexive and motor performance

Measures of simple reflexes are shown in Figure 4. Old mice took longer latencies to initiate walking (Figure 4A) and to turn in a dead-end alley (Figure 4B), 65% and 42% respectively. Treatments had an effect in old mice for walk initiation test and in both age groups for alley turning. Old ExCon and ExEC mice took 31% and 40% shorter latencies to initiate walking than the age-matched control mice. Furthermore, ExCon and ExEC mice took ~38% (young group) and 32% (old group) shorter latencies to turn than the age-matched control mice.

Two-way ANOVAs on each measure revealed significant main effects of Age and Treatment (all Ps < 0.001) but no interaction between Age and Treatment (all Ps > 0.156).

Performance on the wire suspension test, measured as latencies to tread and to fall is presented in Figure 5. Once suspended from the wire, the old SedCon mice took 27% longer latency to tread and 20% longer latency to fall. Overall, the treatments had no effect on treading or falling from the wire with the exception of the young ExCon mice taking slightly longer latencies to fall. Two-way ANOVAs on each measure indicated significant main effects of Age (all Ps < 0.05), but no effect of treatment or interaction between Treatment and Age (all Ps > 0.077).

Performance on the bridge test measured as the average latency to fall from 4 different bridges over 4 sessions is depicted in Figure 6. Old SedCon mice took 22% shorter latency to fall from the bridge compared to the young SedCon ones. Overall the treatment had no effect on bridge performance, with the exception of the ExCon group exhibiting higher latencies than the SedCon within the old groups. A two-way ANOVA revealed a significant main effect of Age (P <0.001), but no effect of Treatment or an interaction with Age and Treatment (all Ps > 0.222).

Spatial learning and memory

The length of the path taken to locate the hidden platform and the speed to reach the platform are depicted in Figure 7 as measures of efficiency. All mice learned to locate the platform after sufficient training as evidenced by shorter path lengths taken as the number of training session increased (Figure 7A). The effect of testing session was confirmed by a three-way analysis with Session as repeated measure (P < 0.001). Overall, old mice took longer path length than the young ones, as evidenced by a significant main effect of Age (P < 0.001). While there was no overall effect of treatment (P = 0.898), the ExCon and ExEC groups took shorter path length during

sessions 1 and 2 than the controls in both age groups. This observation was supported by a significant interaction between Session and Treatment (P = 0.041). Speed was not affected by either Age or Treatment (All Ps > 0.068; Figure 7B). A test for visual acuity was then run to determine whether vision impacted the performance of the mice on the spatial learning and memory task (last session, VP; Figure 7A and B). There was no effect of age or treatment on the performance (path length) of the mice on the visual task, confirmed by two-way ANOVA (all Ps > 0.48). The speed of the ExCon mice was faster on the last session than the other groups, leading to a main effect of Treatment (P = 0.039).

Accuracy for spatial memory was measured by conducting a probe trial as the last trial of sessions 2, 4, 5, 7, and 9, as measured by the percentage of time a mouse spent in a 40-cm annulus around the platform location (Figure 8). Both young and old mice tested developed a strong bias for the platform location as the time spent in the 40cm area increased over training session. This observation was supported by a main effect of Session using a three-way ANOVA with Session as repeated measure (P < 0.001). Overall, the young mice spent more time in the 40-cm area compared to the old mice. While effects of treatment were minor, it was noticeable that the young ExEC mice spent more time in the 40-cm area than any of the other groups. This observation was reflected with an interaction of Session and Treatment (P = 0.015) following a three-way ANOVA with Session as repeated measure. On session 10, which was a probe trial one week after the last session, there was no effect of age or treatment, supported by lack of main effects or interaction after a two-way ANOVA (all Ps > 0.126).

Discriminated avoidance test

The number of trials to reach the discriminated avoidance criterion during acquisition and subsequent reversal sessions as a function of age and treatment is presented in Figure 9. There

was no age effect in any of the sessions, though all mice performed better as a function of session as evidenced by a decreased in the number of trials taken to reach criterion.

During the acquisition session, there was no effect of age however the treatments had an effect on the performance of the mice. The ExCon and ExEC mice took 16-26% (4 months) and 10-16% (20 months) fewer trials to reach criterion, when compared with age-matched controls. Analysis of the trials to the discriminative component for session 1 did not reveal a significant effect of age or interaction between age and Treatment (all Ps > 0.684), but supported a main effect of treatment (P = 0.001).

During reversal 1, all treatment groups took fewer trials than the SedCon in the young group (18-25%), while the SedEC mice took more trials than the SedCon (30%) in the old group. This outcome was supported by a two-way ANOVA yielding a significant interaction between Age and Treatment (P = 0.019). During the reversal 2 session, there was no effect of age or treatment (all Ps > 0.491)

Tail Suspension test

Performance on the tail suspension test, as measured by immobility is presented in Figure 10. There was no effect of age on the performance of the mice, but there were effects of treatments. While it seems that all treatment decreased immobility time, only the difference with SedEC (25%) in the young reached significance (P < 0.05). These observations were supported by a lack of a main effect of Age or interaction between Age and Treatment, and a main effect of Treatment (P = 0.022).

Oxidative stress markers

Catalase activity was measured in 6 brain regions and is presented in Figure 11. There was no effect of age or treatment in any of the regions in either age group, with the exception of an

increased activity in the hindbrain of young ExEC mice. These observations were supported by a lack of main effect or interaction between Age and Treatment following a two-way ANOVA (all Ps > 0.05).

The ratio of GSH to GSSG was calculated and is presented in Figure 12. There was no effect of age or treatment in any of the regions in either age group. These observations were supported by a lack of main effect or interaction between Age and Treatment following a two-way ANOVA (all Ps > 0.05).

Inflammatory markers

Concentrations of IL-6 and TNF-α were measured in the plasma and are presented in Figure 13. Overall, the levels of IL-6 and TNF-α were increased with age and treatments had little to no effect. This was supported by a two-way ANOVA yielding a main effect of Age for both measures (all Ps < 0.011), and no main effect of Treatment or an interaction between Age and Treatment (all Ps > 0.316).

FIGURES AND LEGENDS

Figure 1: Effect of exercise and/or antioxidant supplementation on body weight (A) and food intake (B) in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=19-21 for body weights and n=2-7 for food intake. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C. # $p<0.05$ compared to age-matched SedCon; *$p<0.05$ compared to young treatment-matched mice.

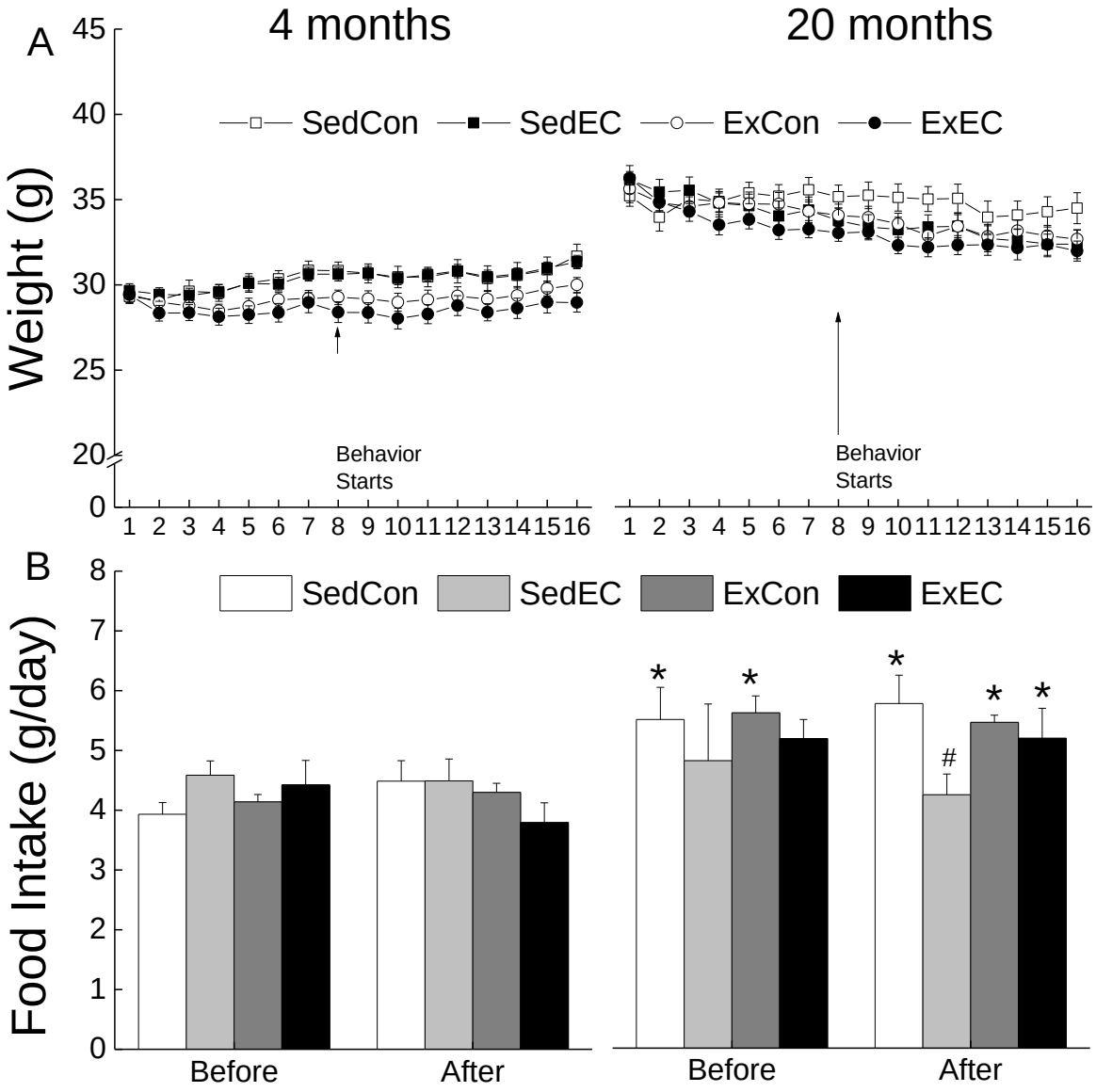

A
4 months
20 months
SedCon
SedEC
ExCon
ExEC
Weight (g)
Behavior Starts
Behavior Starts
B
Food Intake (g/day)
Before
After
Before
After

Figure 2: Effect of exercise and/or antioxidant regimen on locomotor activity as measured by total distance travelled (A), rearing activity (B), and time spent in the center (C) in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=15-18. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C. # $p<0.05$ compared to age-matched SedCon; *$p<0.05$ compared to young treatment-matched mice.

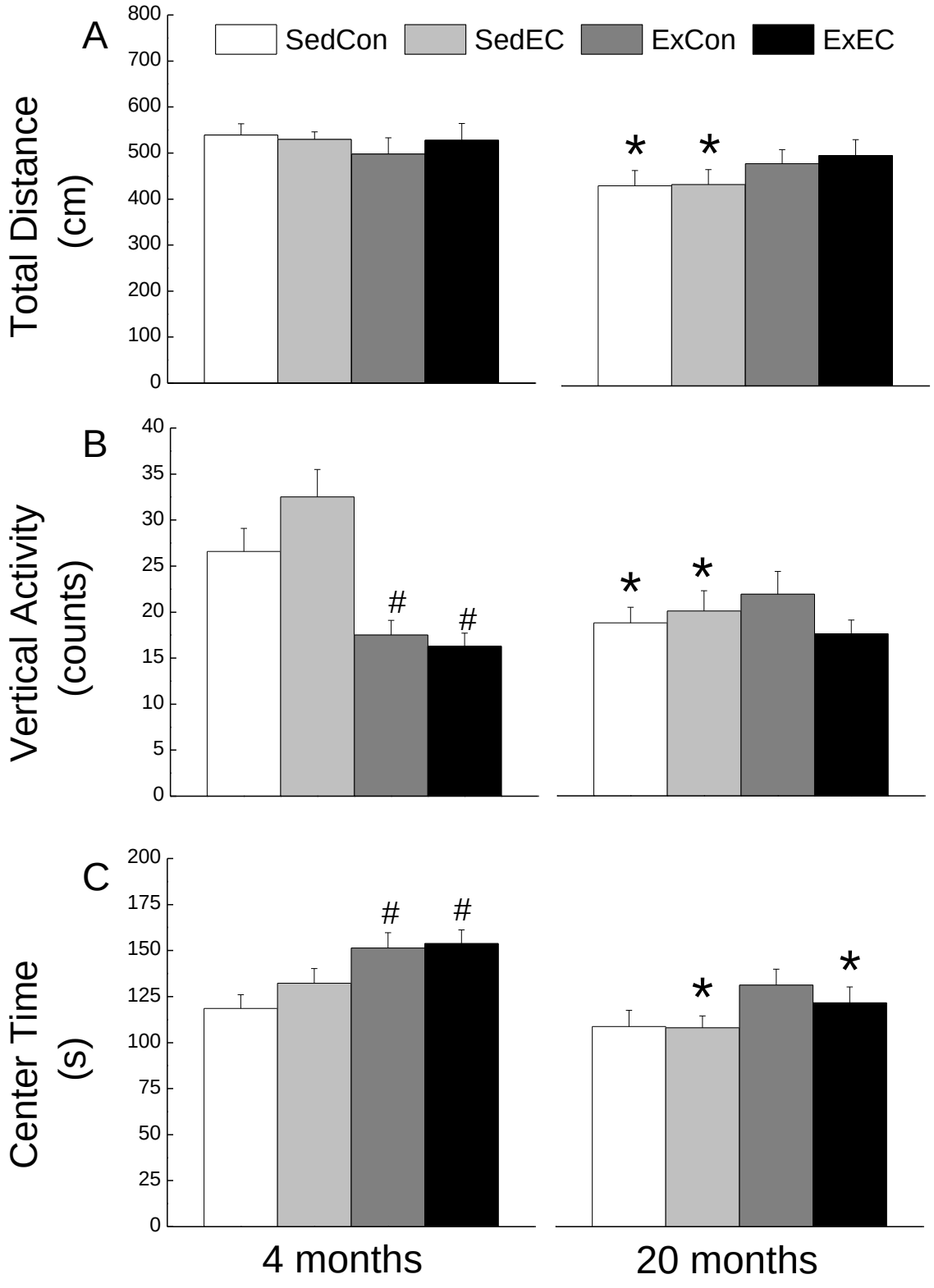

A
SedCon
SedEC
ExCon
ExEC
Total Distance (cm)
800
700
600
500
400
300
200
100
0
*
*
B
Vertical Activity (counts)
40
35
30
25
20
15
10
5
0
#
#
*
*
C
Center Time (s)
200
175
150
125
100
75
50
25
0
#
#
*
*
4 months
20 months

Figure 3: Effect of exercise and/or antioxidant supplementation on motor learning in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=20-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

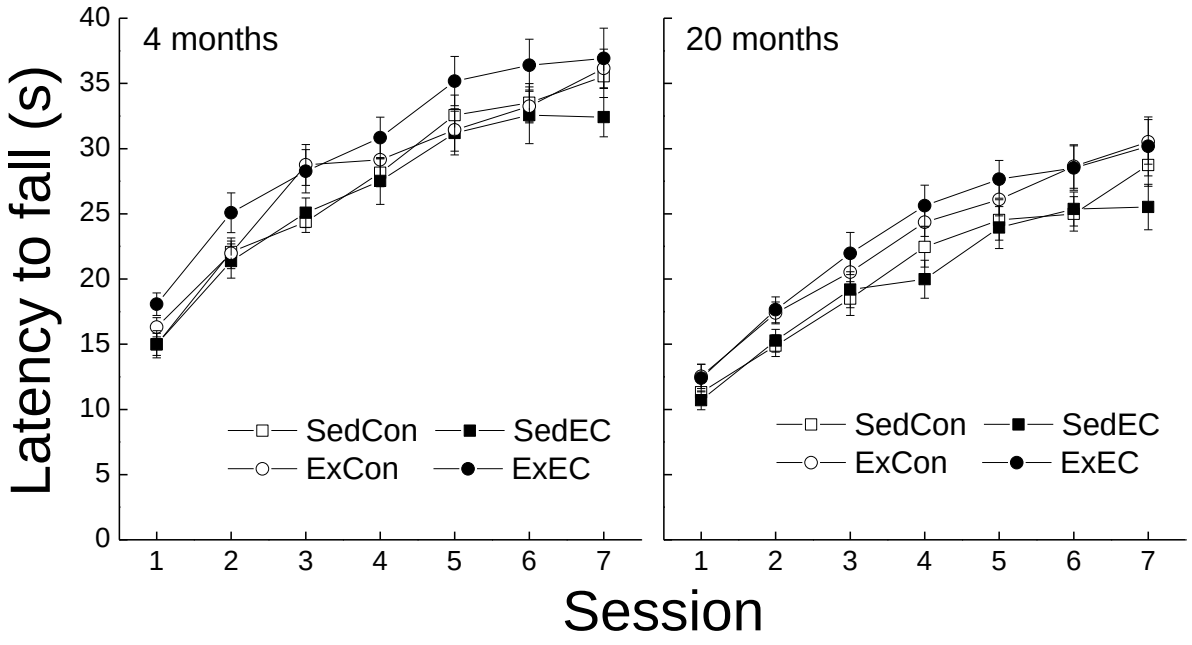

4 months
20 months
Latency to fall (s)
Session
SedCon
SedEC
ExCon
ExEC
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7

Figure 4: Effect of exercise and/or antioxidant supplementation on musculoskeletal reflexive responses as measured by latency to initiate walking (A) and to turn in a dead-end alley (B) in 4- and 20-months C57BL/6J male mice.

Each value represents mean ± SEM, n=20-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

# $p<0.05$ compared to age-matched SedCon; * $p<0.05$ compared to young treatment-matched mice

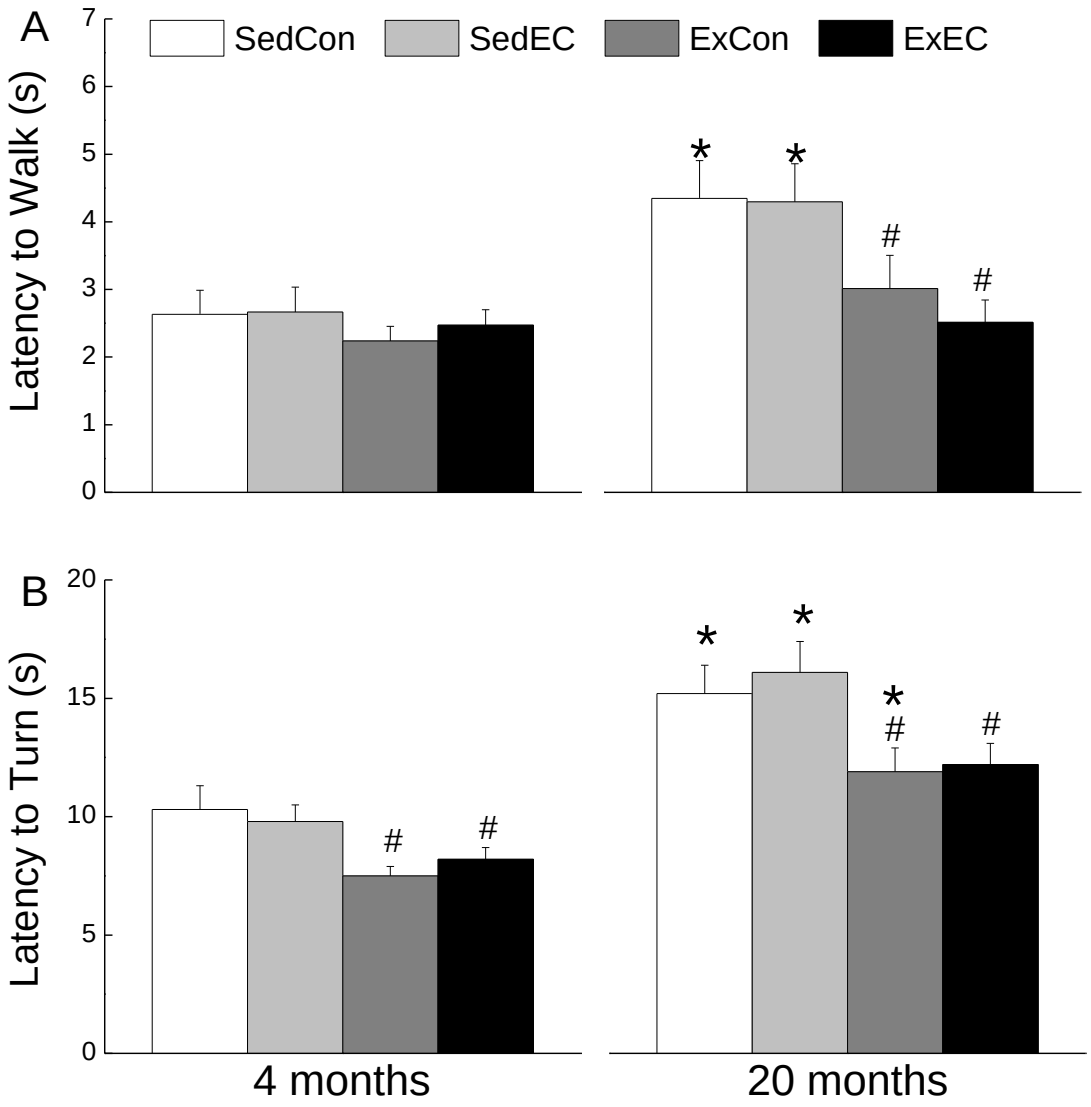

A
SedCon
SedEC
ExCon
ExEC
Latency to Walk (s)
0
1
2
3
4
5
6
7
*
*
#
#
B
Latency to Turn (s)
0
5
10
15
20
#
#
*
*
*
#
#
4 months
20 months

Figure 5: Effect of exercise and/or antioxidant supplementation on performance on wire suspension test as measured by latency to tread (A) and to fall (B) in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=20-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

# $p<0.05$ compared to age-matched SedCon; *$p<0.05$ compared to young treatment-matched mice

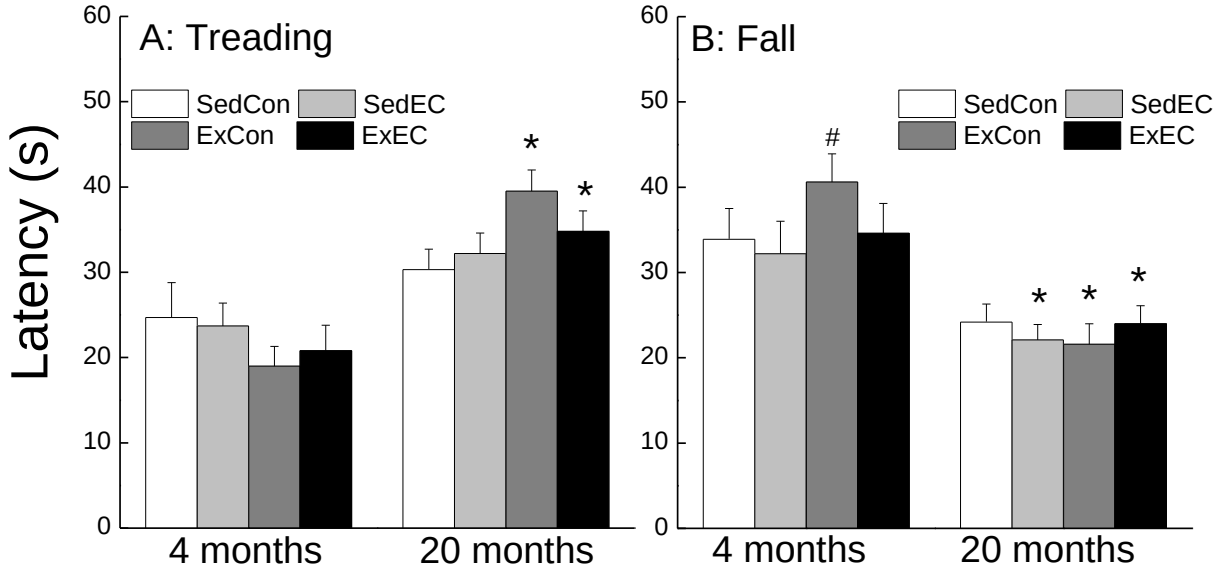

A: Treading
B: Fall
Latency (s)
SedCon
SedEC
ExCon
ExEC
60
50
40
30
20
10
0
*
#
4 months
20 months

Figure 6: Effect of exercise and/or antioxidant supplementation on performance on bridge test in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=20-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

# $p<0.05$ compared to age-matched SedCon; *$p<0.05$ compared to young treatment-matched mice

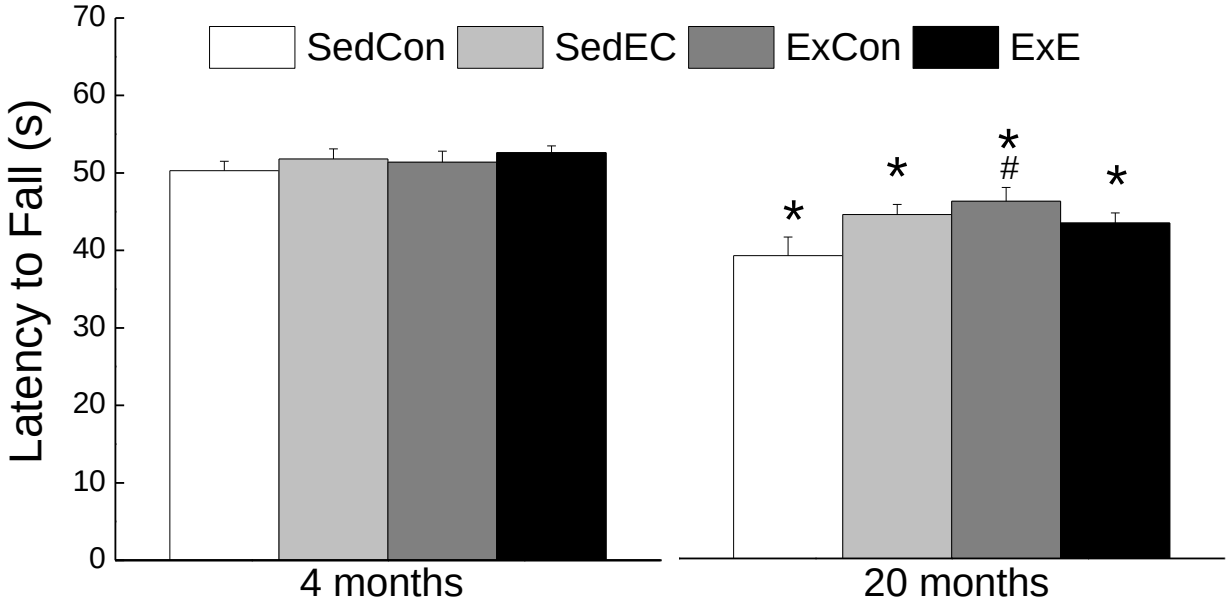

SedCon
SedEC
ExCon
ExE
Latency to Fall (s)
70
60
50
40
30
20
10
0
*
*
*
#
*
4 months
20 months

Figure 7: Effect of exercise and/or antioxidant supplementation on Morris water maze performance as measured by path length (A) and swimming speed (B) in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=19-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

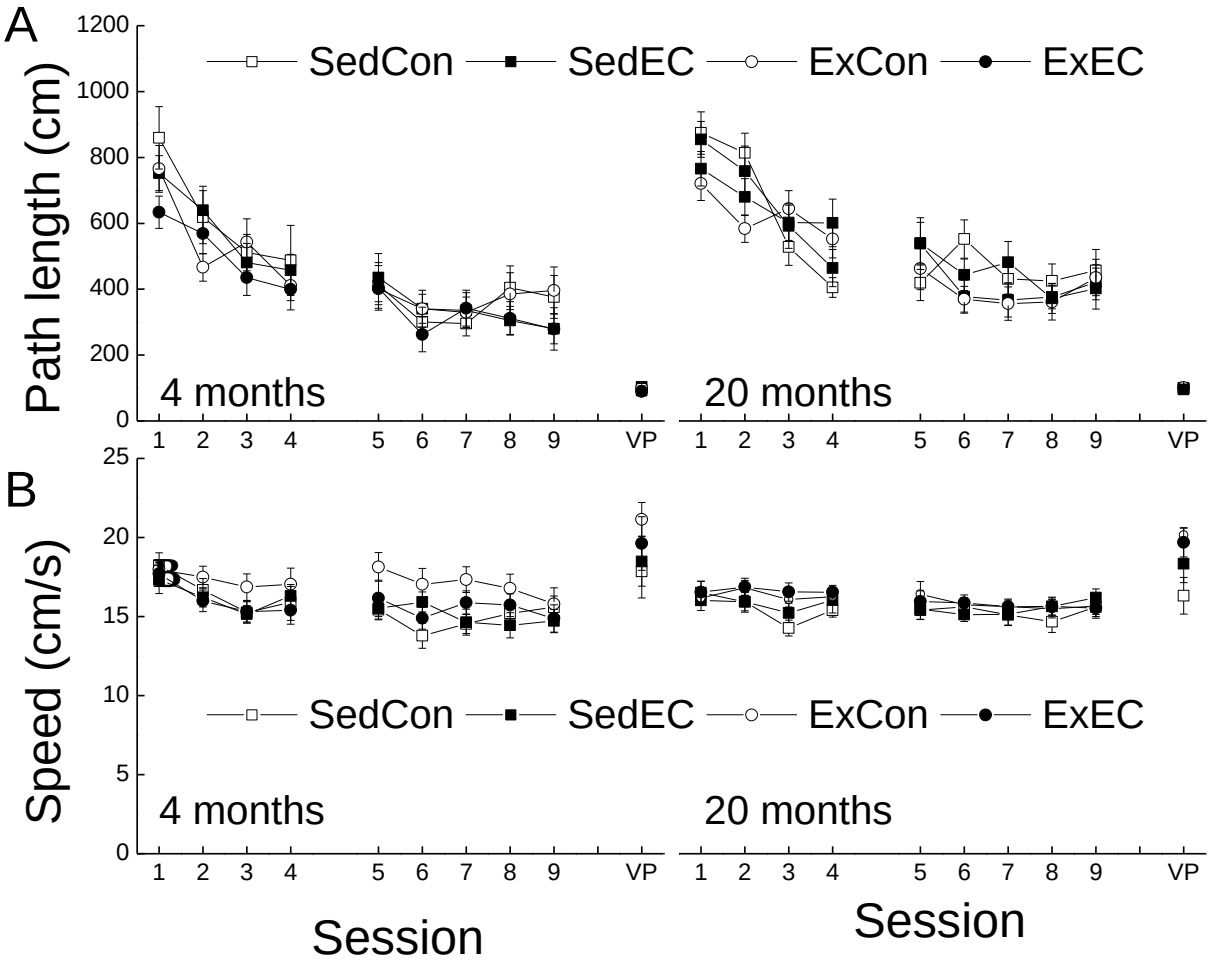

A
Path length (cm)
1200
1000
800
600
400
200
0
SedCon
SedEC
ExCon
ExEC
4 months
20 months
1 2 3 4 5 6 7 8 9 VP
B
Speed (cm/s)
25
20
15
10
5
0
SedCon
SedEC
ExCon
ExEC
4 months
20 months
1 2 3 4 5 6 7 8 9 VP
Session
Session

Figure 8: Effect of exercise and/or antioxidant supplementation on Morris water maze probe trial performance as measured by percent time spent in a 40-cm annulus in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=19-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C. The dotted line represents the percent time a mouse would be in the 40-cm annulus due to chance.

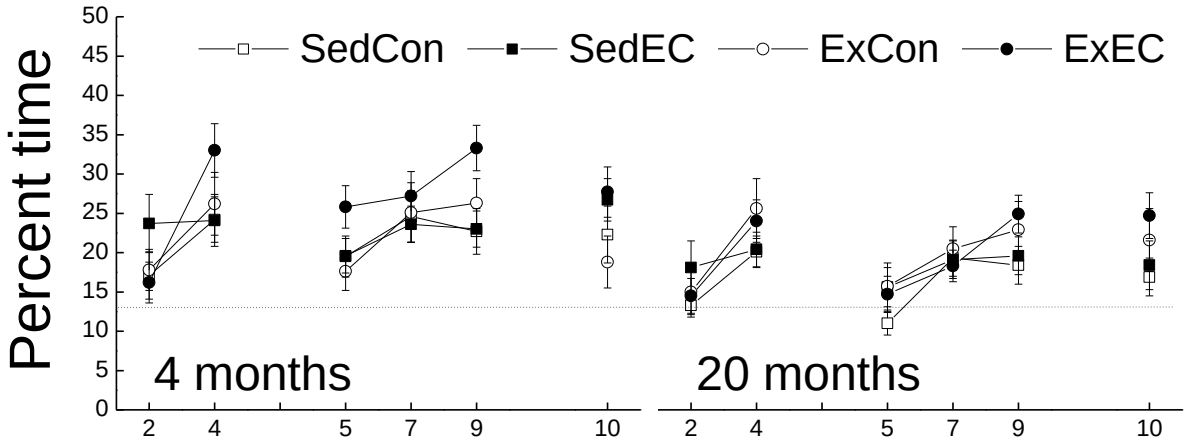

Percent time
SedCon
SedEC
ExCon
ExEC
4 months
20 months
0
5
10
15
20
25
30
35
40
45
50
2
4
5
7
9
10

Figure 9: Effect of exercise and/or antioxidant supplementation on discriminated avoidance performance, measure by the number of trials taken to reach criterion during acquisition session and 2 reversal sessions in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=17-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

# $p<0.05$ compared to age-matched SedCon; *$p<0.05$ compared to young mice

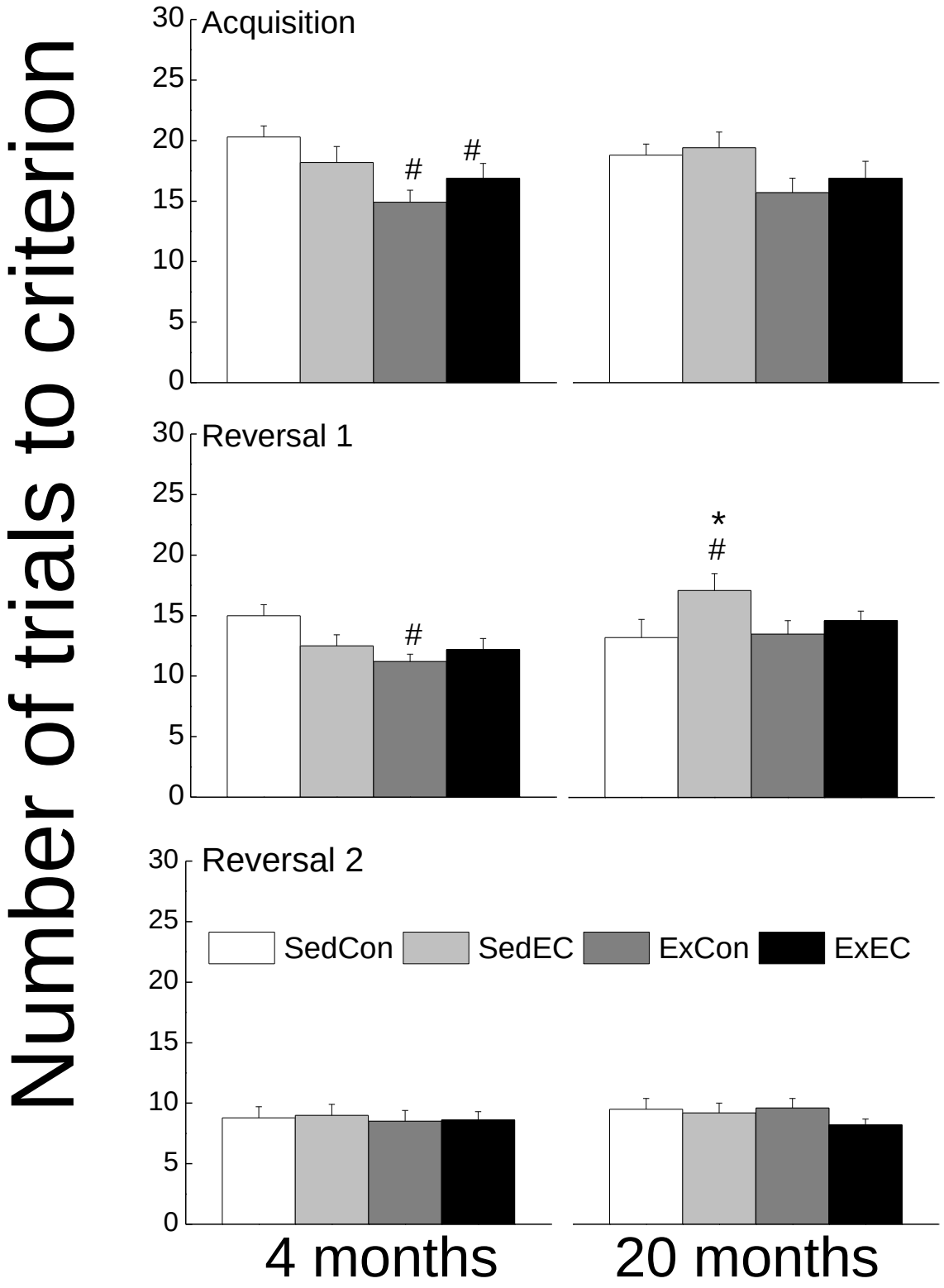

Number of trials to criterion
Acquisition
Reversal 1
Reversal 2
30
25
20
15
10
5
0
#
#
#
*
#
SedCon
SedEC
ExCon
ExEC
4 months
20 months

Figure 10: Effect of exercise and/or antioxidant supplementation on suspension test performance, measure by immobility time in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=17-21. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

# $p<0.05$ compared to age-matched SedCon.

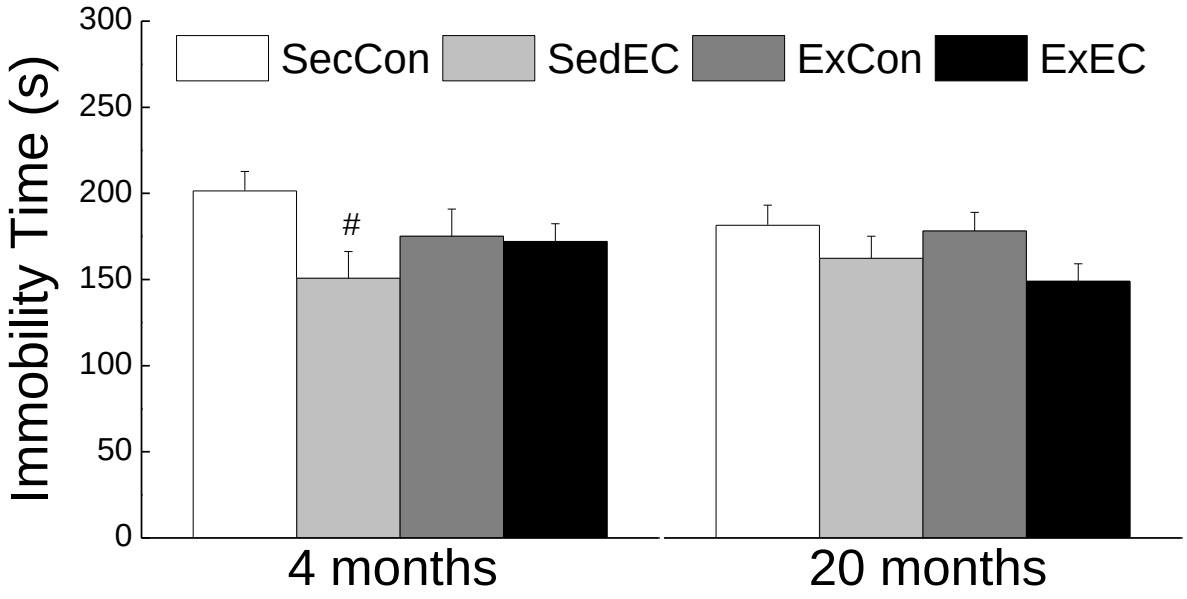

Immobility Time (s)
300
250
200
150
100
50
0
SecCon
SedEC
ExCon
ExEC
#
4 months
20 months

Figure 11: Effect of exercise and/or antioxidant supplementation on catalase activity in 6 brain regions in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=6-7. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

# $p<0.05$ compared to age-matched SedCon.

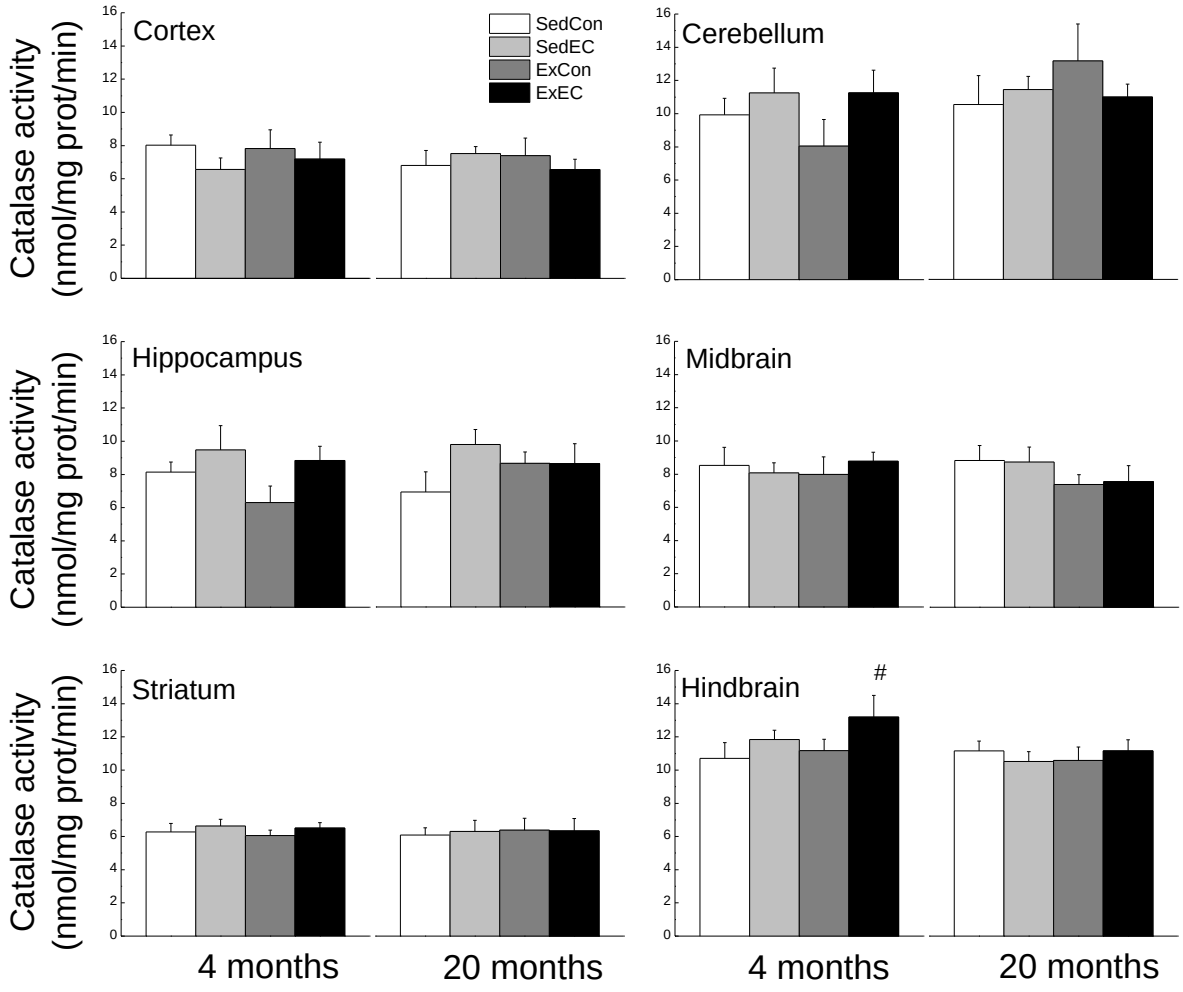

Cortex
SedCon
SedEC
ExCon
ExEC
Cerebellum
Hippocampus
Midbrain
Striatum
Hindbrain
#
Catalase activity (nmol/mg prot/min)
4 months
20 months

Figure 12: Effect of exercise and/or antioxidant supplementation on GSH/GSSG ratio in 6 brain regions in 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=3-6. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

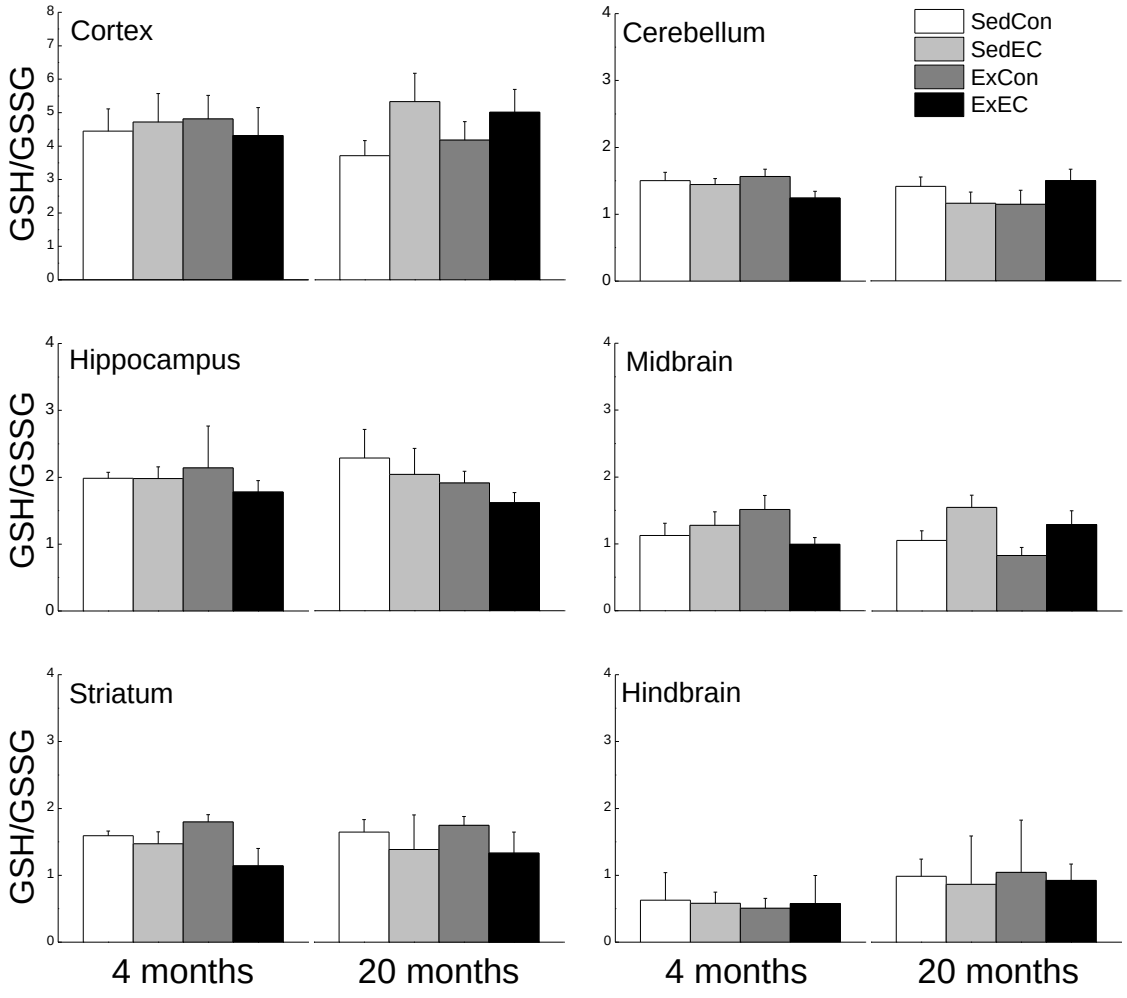
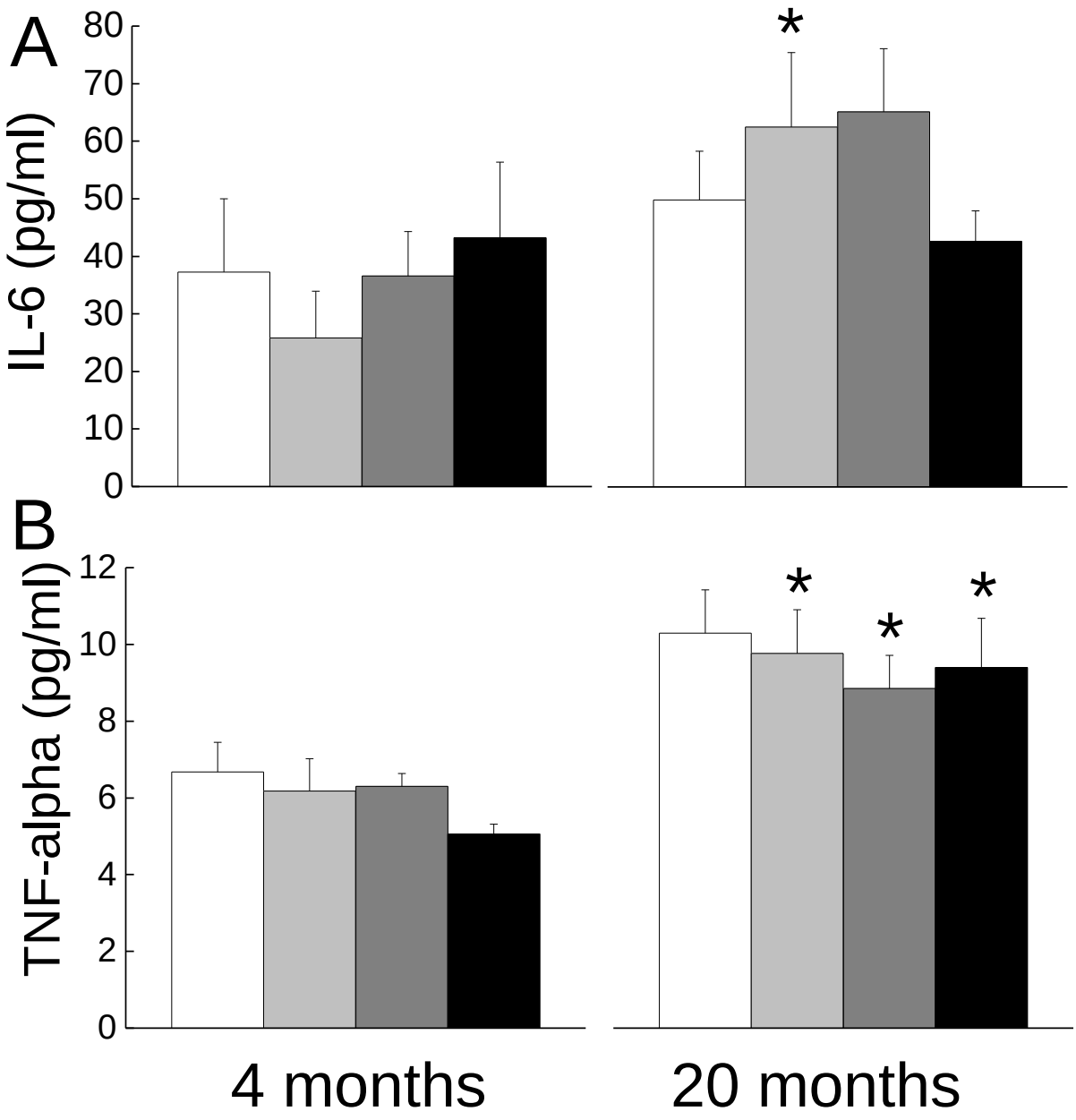

Cortex
Cerebellum
Hippocampus
Midbrain
Striatum
Hindbrain
GSH/GSSG
SedCon
SedEC
ExCon
ExEC
4 months
20 months
4 months
20 months

Figure 13: Effect of exercise and/or antioxidant supplementation on inflammatory markers in the plasma of 4 and 20-months old C57BL/6J male mice.

Each value represents mean ± SEM, n=3-6. SedCon, sedentary fed the control diet; SedEC, sedentary fed the control diet enriched with vitamins E and C; ExCon, exercise fed the control diet and ExEC; exercise fed the control diet enriched with vitamins E and C.

*p<0.05 compared to young treatment-matched mice


A
IL-6 (pg/ml)
80
70
60
50
40
30
20
10
0
*
B
TNF-alpha (pg/ml)
12
10
8
6
4
2
0
*
*
*
4 months
20 months

CHAPTER 4

DISCUSSION

This study confirmed the existence of robust age impairments in several aspects of reflexive, motor and cognitive functions. Antioxidant supplementation did not reverse any age-related dysfunction, rather worsened cognitive flexibility of old mice. Interestingly, antioxidant supplementation decreased depression-like behavior in young mice. Moderate exercise was more successful in improving motor and cognitive function, and some improvements were age-specific. Most importantly, the combination of exercise and antioxidant did not yield additional beneficial effects. Noteworthy, no negative interaction between the two treatments was observed.

Short-term treatment with antioxidants and exercise for 16 weeks in mice, which translates to approximately 15-20 years in humans. The present study suggests that regular antioxidants and exercise for number of years is essential for improvements in brain function at an advanced age.

The young mice gained weight during the period of the study whereas the old group lost weight. These results are in accordance with previous findings for C57BL/6 mice (Sumien et al. 2004; Sumien et al. 2009; Baynard et al. 2012). The present data suggests that, exercise training did not affect weight gain in young mice or weight loss in old mice, validating that the intensity of exercise form was indeed moderate. Similarly, animal and human studies have revealed that moderate intensity exercise alone does not lead to weight loss(Caudwell et al. 2009; Baynard et al. 2012). While a voluntary exercise paradigm may seem more appealing than a forced regimen, it is noteworthy that voluntary wheel running involves higher exercise speed and duration (Leasure and Jones 2008a). Moreover, forced exercise paradigm unlike voluntary wheel running does not lead to caloric deficit resulting in weight loss (Bell et al. 1995; Baynard et al. 2012).

Interestingly, the food intake in the exercise group did not increase even though the mice had ad libitum supply of food throughout this study. Our findings align with previous studies that utilized moderate exercise paradigm compared to strenuous exercise which generally lead to

weight loss and a corresponding increase in food intake (Bell et al. 1995; Mainardi et al. 2010; Baynard et al. 2012). Interestingly, in the present study vitamin E and C supplementation was associated with a significant decrease in food intake in old mice. In contrast, in a previous study antioxidant intake lead to higher food intake in middle-aged Apolipoprotein E (*APOE)*-3; suggesting strain differences (unpublished data). However, food intake was done within group housing setting and may explain some of the discrepancy between food intake and also lessen the power of the studies by having less measurements (1 per cage instead of 1 per mouse).

Antioxidant supplementation is one of the most popular strategies used to attenuate age-associated functional deficits (Kaikkonen et al. 1998; Shukitt-Hale et al. 1999; Fusco et al. 2007). Based on the human:mouse body surface area ratio of 12.3, the human dose equivalent of vitamins used in the present study correspond to 800 IU of Vitamin E and 1,150 mg of Vitamin C (Reagan-Shaw et al. 2008). The doses of vitamin E and C in the present study were selected on the basis of preliminary studies conducted in our laboratory, that demonstrated a reversal of age-related functional impairments in old mice (unpublished data). Furthermore, doses used in the present study have been widely tested in clinical studies, and are significantly higher than the current recommended dietary allowance (RDA) of vitamin C (60 mg/d of for adult nonsmoking men and women), and vitamin E (22.4 IU/day) ((Martin et al. 2002; Cetin et al. 2010; Li et al. 2012)NIH fact sheet, Office of Dietary Supplements 2013). Previous study in our lab found that concentrations of α-tocopherol following supplementation with this test diet were increased 3-5 fold in plasma and tissue homogenates in old C57BL6 mice after 13 weeks of dietary supplementation (Sumien et al. 2003, 2004). The present study did not measure the levels of vitamin C in C57BL6 mice following vitamin C supplementation. In humans, oral high doses vitamin C shows lower bioavailability and saturates plasma and tissue (Levine et al. 1996). In

alignment with previous studies, the present study demonstrates an age-associated decline in the psychomotor and cognitive function measured by standard tests of psychomotor functions such as balance (bridge-walking), coordinated running (rotorod), muscle strength (wire suspension), reflexive ability (walk initiation and alley turn), and spatial memory (water maze)(Sumien et al. 2004; Sumien et al. 2006; Shetty et al. 2013b). However, the present study did not observe significant effect of age on learning and cognitive flexibility measured during the discriminated avoidance task. It is important to note that when tested on the discriminated avoidance, the mice were older (8 and 24 months vs. 4 and 20 when the study was initiated). Previous studies have shown that some memory deficits can occur early, and that at 8 months of age the mice are already impaired (Shukitt-Hale et al. 1999; Sumien et al. 2004; McDonald and Forster 2005) .

Furthermore, human and animal studies suggest an increase in prevalence of depression with aging (Beekman et al. 1999). However, the present study failed to observe an effect of age on the tail suspension test. Similar to our findings, Malatynska et al. found that aged C57BL/6 mice exhibit hedonic deficits similar to depressed elderly humans, but had relatively intact affective behaviors, measured by forced swim and tail suspension test. Also, aging did not affect the immobility time of 15 months old CC57Br, A/He and C3H/He but lead to increase in floating time of 20- to 24-month-old BALB/c mice treated with lipopolysaccharide in forced swim test, suggesting strain differences (Skrinskaia and Nikulina 1994). Compared to healthy individuals, patients with major depression have significantly lower serum levels of Vitamin E (Maes et al. 2000; Owen et al. 2005; Mazloom et al. 2013) and Vitamin C (Khanzode et al. 2003). Similar to previous reports, the present study showed a beneficial effect of Vitamin E and C supplementation on depression-like behavior on tail suspension test performance. Similarly, Lobato et al. found that

acute or chronic treatment with α-tocopherol reduced the immobility time in the tail suspension and forced swim test (Lobato et al. 2010).

In contrast to previous studies, high doses of vitamins E and C did not seem to improve psychomotor or cognitive function in aged mice, but improved simple reflexes in alley turn and alleviated depression-like symptoms in tail suspension test in young mice. In a previous study, short-term Vitamin E supplementation (alpha-tocopheryl acetate, 1.65 g/kg) also failed to improve coordinated running, bridge walking or swim maze performance of 20-month-old C57BL6 mice (Sumien et al. 2004). Another study found that 16 weeks of antioxidant treatment (phenyl-α-tert-butyl nitrone, α-tocopherol, and ascorbate) in old rats improved cognitive function but failed to improve motor function , (Socci et al. 1995). In addition, short-term treatment with vitamin C alone failed to improve the cognitive function of 15 months old mice (Arzi et al. 2004). Also, few clinical studies involving Parkinson's disease (PD) patients failed to detect the efficacy of vitamins E and C in altering motor or cognitive performance, but delayed the need to use drugs in PD patients (Fahn 1991; Fahn 1992). An unanticipated finding was that vitamins E and C intake worsened the discriminative avoidance test performance in the reversal session in old mice.

In the present study, antioxidants did not have a favorable outcome on the brain function. It is possible that neurons may have died or synapses may have been lost in the old age, when antioxidant treatment was started (Petersen et al. 2005). Also, recent studies have also emphasized the benefits of initiating antioxidants supplementation at an earlier age to prevent age-related impairments as it is difficult to reverse already existing age-related damage (Shukitt-Hale et al. 1999; Sumien et al. 2004; McDonald and Forster 2005). Furthermore, some evidences suggest that prolonged administration of vitamin E ameliorates age-associated cognitive dysfunction and reduce oxidative damage in the brain (McDonald and Forster 2005).

Furthermore, the exercise protocol used in the present study has similar training effect in young and older mice (an equivalent increase in activity of citrate synthase in rectus femoris), that is also comparable to that observed in human studies (unpublished data). Furthermore, a kinetic analysis based on stride length of mouse vs. human suggested that the regimen used in this study would be similar to a brisk walk of ~3.4 miles in one hour, which is considered to be moderate type. The intensity of exercise can be predicted by measuring factors such as heart rate, oxygen uptake, and perceived exertion (Duncan et al. 2001; Dunbar and Kalinski 2004).

The present data shows that exercise leads to an improvement of reflexes in mice irrespective of age. Previous study in our lab also found an improvement in the reflexes of APOE3. Similar to our findings, physical activity for 20 weeks improved the reaction time of older individuals (Lord and Castell 1994). Exercise-induced improvements in the reflex behavior may be due to an increase in cerebral circulation and the alteration of the action of neurotransmitters (Kashihara et al. 2009).

The present data suggests that exercise in young mice seemed to decrease rearing in spontaneous activity test. Similar results were found in an earlier study using young APOE 3 mice (unpublished data). Furthermore, the exercise mice seemed to spend more time in the center of the spontaneous activity apparatus, reflecting a lower degree of anxiety. These findings are consistent with earlier observations where moderate treadmill exercise reduced anxiety-like behaviors in mice and rats (Salim et al. 2010; Chaudhari et al. 2014). However, one study observed that forced but not voluntary exercise increased anxiety-like behaviors of Long-Evans rats in the open field test. It should be noted that forced exercise regimen did not involve a treadmill based, but motorized wheels atop rotating axles were used (Leasure and Jones 2008a).

Furthermore, in the present study exercise improved grip strength only in young mice. Similarly, single treadmill walking session for 20 minutes improved the grip strength of young men with Down syndrome (Chen et al. 2014a). However, in contrast other studies did not find an effect of exercise on strength. To exemplify, Leiter and colleagues found no effect of voluntary wheel running for 3 weeks on grip strength in young (8 months) and old (18 months) C57BL/6 female mice using calibrated strain gauge (Leiter et al. 2012). In addition, in a clinical study Moritani and deVries reported that eight weeks of strength training did not improve strength gains of old and young men (Moritani and deVries 1980; Lambert and Evans 2002). In our view, the inconsistency in these results might be due to the difference in factors such as species, age, sex, intensity and duration of exercise, and choice of behavioral tests.

In a previous study, exercise improved the balance of young APOE3 mice but did not affect the performance of old mice (unpublished data). In contrast, the present data suggests that exercise reversed age-related declines in balance of old but not young mice. Similarly, Pothakos et al. found that 10 weeks of treadmill based exercise effectively improved balance and gait performance of C57BL/6 mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/probenecid (Pothakos et al. 2009). Clinical studies have also confirmed the benefits of exercise in PD patients (Miyai et al. 2000; Chen et al. 2005). Furthermore, a recent study found that exercise significantly improved the balance of osteoporotic women older than 70 years of age (Vaillant et al. 2006). Similarly, exercise training for 6 weeks in older diabetic patients improved the balance, proprioception, lower-limb strength, reaction time, and, consequently, decreased risk of falling (Morrison et al. 2010). A recent study by Gorton et al. found that treadmill exercise significantly increased rotorod performance of vehicle treated young adult male C57BL/6 mice (Gorton et al. 2010), consistent with our findings. However, it should be noted that the improvements with

exercise were marginal on motor learning and gross motor function, but were rather noticeable on reflexes.

Human and animal studies have demonstrated that regular physical activity has been associated with an improvement in cognitive function (Kramer et al. 1999; Sutoo and Akiyama 2003; Berchtold et al. 2005). The present study data demonstrates that exercise improved the discriminative avoidance performance of young mice. Similarly, Chaudhari et al. reported that exercise improved learning in acquisition and reversal sessions of young APOE3 mice (Chaudhari et al. 2014). A growing literature suggests that exercise training is associated with improvement in passive avoidance learning (Saadati et al. 2010). Also, exercise training reversed morphine impaired the short-term memory and learning (Alaei et al. 2006). Together these findings suggest that exercise training is associated with an improvement in associative learning and cognitive flexibility in mice.

While, there are studies that demonstrated that both voluntary and forced exercise regimen improve hippocampal-dependent cognitive function (Berchtold et al. 2005; O'Callaghan et al. 2007; Berchtold et al. 2010; Lin et al. 2012), Barnes et al. reported that there was no effect of forced exercise training for 10-week on spatial learning in aged rats (Barnes et al. 1991). In the current study, effects on spatial learning were minimal at best. However, it should be noted that the combination of exercise and antioxidants increased spatial accuracy of the young mice during MWM.

The data on combination of antioxidant and exercise is very limited and equivocal. Aged rodents on antioxidant rich diet and swimming exercise have reported an additive effect on markers of oxidative stress in various brain regions (Devi and Kiran 2004; Wu et al. 2008). Whereas, few studies observed an antagonistic action of antioxidants on the beneficial effects of exercise (Ristow

et al. 2009; Paulsen et al. 2014). Taken together, it appears that interaction between antioxidant supplementation and exercise is highly complex and may depend on multiple factors.

Similar to the exercise group, the combination antioxidant intake and exercise lead to improvement in reflexes, motor and cognitive performance in young and/or old mice. Interestingly, the coordinated running performance of the young mice was improved in the learning phase before a plateau was reached. However, all these effects were significant, combining antioxidants to exercise did not lead to additive effects. These findings were in alignment with a previous study conducted in our lab using APOE 3 and APOE 4 mice (Chaudhari et al. 2014). Similarly, recent clinical studies reported that vitamin C and vitamin E supplementation failed to improve physical performance in well-nourished individuals (Gerster 1989; Williams 1989; Tiidus and Houston 1995; Williams 2004). It is possible that maximal ceiling effect in the performance was reached by individual interventions, thus further improvement in the behavior was not observed. However, further studies are needed to better understand the complex molecular mechanisms of interaction between exercise and antioxidant supplements.

Recent studies have suggested that when implemented independently, antioxidant supplementation and exercise can improve psychomotor and cognitive function, by attenuating age-associated increases in the oxidative damage in brain (Somani et al. 1995; Elokda and Nielsen 2007). Therefore, it is anticipated that combination of these interventions will effectively improve the cellular redox status. Unexpectedly, the intake of antioxidants or exercise failed to improve GSH:GSSG ratio or increase the level of catalase enzyme in different regions of the mouse brain, even though an improvement in behavior was observed.

Similar to present data, a previous study found that regional differences in the glutathione levels in each specific brain region and cerebral cortex had higher levels of GSH than other regions

(Somani et al. 1995). Also, exercise training or antioxidants did not significantly change GSH levels in this region (Somani et al. 1995). Similar to present study, Ozkaya et al. found that exercise did not alter catalase activity of brain of non-diabetic rats (Ozkaya et al. 2002)

Some other studies have suggested that beneficial effects of exercise on brain function are mediated via improvement in redox status, angiogenesis (Kerr et al. 2010), neurogenesis (van Praag et al. 1999; Brown et al. 2003; Pereira et al. 2007). Furthermore, exercise has been associated with upregulation of neuroplasticity signaling factors such as AKT, CREB phosphorylation and BDNF in the hippocampus and thus contribute to maintenance of cognitive function during aging (Vaynman et al. 2004; Berchtold et al. 2005; Aguiar et al. 2011; Liu et al. 2013).

In a recent study by Aguiar et al. intense exhaustive exercise regimens for 8 weeks elevated TBARS levels and reduced BDNF cortical levels, demonstrating mitochondrial dysfunction in brain of young male mice (Aguiar et al. 2008). However, 10 days of intense exercise in young male C57Bl/6 mice, lead to memory reduction of exercised animals in comparison with the control group, and an associated increase in the brain oxidative stress (Rosa et al. 2007). Conversely, in 20 months old rats over-training did not induce oxidative stress in the brain or caused loss of memory, indicating the significant role of age in determining the outcome (Ogonovszky et al. 2005).

Furthermore, in alignment with previous studies the present study found an age-dependent increase in the levels of IL-6 and (TNF)-α (Pedersen et al. 2000; Bruunsgaard and Pedersen 2003). However, in contrast to previous studies antioxidants and exercise failed to affect the levels of plasma pro-inflammatory markers (Colbert et al. 2004; Yfanti et al. 2010).

Overall, exercise training alone and in combination with antioxidants, but not antioxidants alone, reversed age-related declines in motor and cognitive function. Furthermore, antioxidants

supplementation did not block the beneficial effects of exercise on age-associated behavioral decline in psychomotor and cognitive function.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

This dissertation work aimed to investigate whether the combination of antioxidants with exercise will maximally reverse age-related psychomotor and cognitive dysfunction. The present study demonstrated that antioxidant supplementation and moderate exercise improved various aspects of psychomotor and cognitive function in both young and old mice. However, the combination of exercise and antioxidant did not lead to additive or antagonistic effects.

It is possible that maximal ceiling effect in the performance was reached by individual interventions, thus further improvement in the behavior was not observed. However, further dose response studies are needed to better understand the complex interaction between exercise and antioxidant supplements.

Furthermore, lifestyle modifications factors such as exercise and antioxidant supplementation are increasingly being recommended as an important strategy to counteract the deleterious effects of age on brain function. However, selecting the accurate type, duration, and frequency of exercise or antioxidants has been challenging; especially in the elderly. Therefore, depending on the intended outcome these parameters may need to be adjusted. The results from this work may provide guidance for physicians to prescribe optimal doses of antioxidant and customized exercise regimens to delay or slow the cumulative functional declines associated with the aging process. The present results will also provide scientific evidence for designing clinical trials intended to evaluate effects of antioxidant supplementation and exercise training in elderly.

Furthermore, aging research should strive to understand the mechanisms responsible for the beneficial effects of exercise and develop new drugs that are capable of mimicking those complex mechanisms. Nevertheless, it is equally important to understand various interactions with other interventions; especially when it can be negative. The implications of exercise-mimetics are

not just limited to elderly individuals, but also extend to the patients who cannot exercise due to various circumstances.

This work corroborates the findings of studies that observed functional benefits of moderate exercise on brain function and provide a foundation of experiments that study the interaction of exercise with antioxidants; especially in aged mice. However, many new areas are yet to be explored. A mentioned earlier that there is possibility that maximal ceiling effect in the performance was attained by individual interventions. To avoid the ceiling effect, one avenue that could be explored is to study the effects of different regimens such as intermittent antioxidant and moderate exercise regimen on the psychomotor and cognitive function. It is also important to study the sex differences in the context of the present study and further explore the interaction between exercise and antioxidant supplementation in females. Since, oxidative stress is more in pre-menopausal females compared to males, it is expected that interaction between antioxidants and exercise may result in differential effects on brain function.

One of the limitations of this study was that glutathione, which was determined by Cayman's kit, which does not seem to measure the levels accurately in the brain tissue as compared to a more robust technique; high-performance liquid Chromatography (HPLC). Furthermore, the present study evaluated limited markers of oxidative stress such as catalase activity and GSH: GSSG ratio. Also, we believe that additional biochemical tests such as Malondialdehyde (MDA), Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and selenium-dependent glutathione peroxidase (GSPHx) could be considered to establish the role of oxidative stress in aging and effects of treatment . Since, we estimated oxidative stress parameters in the entire brain regions, it is possible that might have diluted the effects of treatments and aging. We suggest using immunohistochemistry methods for the detection and localization of oxidation in the brain.

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