DISSERTATION

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Effect of one year vitamin E and C supplementation on elderly with Mild Cognitive Impairment (MCI) in Isfahan, Iran

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Dedication

To My wife for her love and devotion

To my lovely children Pouya and Parto

To my family for their encouragement

In memory of my father
ABBREVIATIONS

8-OHdG: 8-hydroxydeoxyguanosine

Aβ: β-amyloid

AD: Alzheimer’s Diseases

AOX: Antioxidant

APOE ε4: Apolipoprotein E ε4

BMI: Body Mass Index

BP: Blood Pressure

CBC: Cell Blood Count

CDR: Clinical Dementia Rating

CI: Confidence Interval

CSF: Cerebrospinal Fluid

CVD: Cardiovascular Disease

DNA: Deoxyribonucleic Acid

DTNB: 5, 5’-dithiobis -2-nitrobenzoic Acid

EDTA: Ethylenediaminetetraacetic Acid

ELISA: Enzyme-linked Immunosorbent Assay

EFSA: European Food Safety Authority

GPx: Glutathione Peroxidase

GR: Glutathione Reductase

GSSG: Glutathione Disulfide

H2O2: Hydrogen Peroxide
**Hb:** Hemoglobin

**HPLC:** High Performance Liquid Chromatography

**MCI:** Mild Cognitive Impairment

**MDA:** Malondialdehyde

**MMSE:** Mini Mental State Examination

**NFT:** Neurofibrillary Tangles

**O$_2^-$:** Superoxide

**OH:** Hydroxyl Radical

**OS:** Oxidative Stress

**RDI:** Recommended Daily Intake

**RNS:** Reactive Nitrogen Species

**ROS:** Reactive Oxygen Species

**SEM:** Standard Error of the mean

**SOD:** Superoxide Dismutase

**SP:** Senile Plaques

**TAC:** Total Antioxidant Capacity

**TAS:** Total Antioxidative Status

**TBA:** Thiobarbituric Acid

**TBAR-S:** Thiobarbituric Acid Reactive Substances

**TNB:** 2-nitro-5-thiocyanobenzoic Acid

**Vitamin C:** Ascorbic Acid

**Vitamin E:** DL-α-tocopherol acetate
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1. INTRODUCTION

Dementia is a general term that refers to a number of progressive disorders which lead to decline in memory, judgment, decision making, learning, executive functioning and other mental activities. These disorders mainly affect older people, although there is a raising awareness of cases that start before the age of 65. In 2010, an estimated 35.6 million people were diagnosed with dementia throughout the world; the number is expected to increase to 115.4 million by 2050. Almost, two-thirds of the patients live in low and middle income countries, where the most increases in number of patients is anticipated (Wimo and Prince, 2010).

Alzheimer’s disease as the most known cause of dementia in elderly individuals is described by progressive neurodegenerative modification that gradually reduces cognitive and functional abilities of the patients (Mecocci and Polidori, 2012). Alzheimer’s disease may occur after a long stage of neuropathological alterations and cognitive decline, known as Mild Cognitive Impairment (MCI) (Baldeiras et al, 2008).

MCI, without significant effect on the daily activities, is a term to describe the transitional state between normal aging and Alzheimer’s disease.

The criteria for MCI include: 1) memory complaint, 2) age-associated memory impairment, 3) normal general cognitive status, 4) normal activities of daily living, 5) absence of dementia, (Nelson and O’Connor, 2008).

Elderly individuals with MCI have clinically significant memory impairment, often along with the functional deficits in attention, speech, decision-making, language, visuospatial and psychomotor function. The severity of symptoms is greater than expected for their age and educational level but symptoms are not severe enough to meet the criteria for dementia (Chen et al, 2001; Petersen et al, 2001).

The MCI patients have a significantly higher rate of progression to AD (12 to 15 % per year) in comparison with cognitively normal elderly people (1 to 2% per year) (Padurariu et al, 2010; Smith et al, 1996).
Currently, Researchers in the field of MCI focus on identifying the risk factors of disease progression for the purpose of early treatment intervention, which may delay or even prevent the disease process (Huang C, 2003).

Numerous evidences indicate that oxidative stress plays a major role in the pathogenesis of AD and it occurs early in the disease. Oxidative damage implies the development of reactive oxygen species (ROS). It can change the structure and function of cellular key components. Oxidative stress is an imbalance in pro-oxidant/antioxidant homeostasis that leads to the generation of toxic reactive oxygen species. Cerebral tissue is more susceptible to ROS damage due to high oxygen requirements for metabolism, low content of antioxidants and a high content of polyunsaturated fatty acids (Padurariu et al, 2010; Baldeiras et al, 2008; Lovell and Markesbery, 2008). Brain cells are continuously exposed to ROS produced by oxidative metabolism, and defense mechanisms against oxygen radicals may be weakened and/or overwhelmed in the certain pathological conditions (Berg D, 2007).

For instance, it has been reported that the endogenous antioxidant enzymes including Superoxide Dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase, reduce activities in the AD brains (Keller et al, 2005). Many studies have also provided evidence for the oxidative damage in the brains of patients with Alzheimer’s disease including the presence of increased levels of oxidized macromolecules such as lipids, nucleic acids, and proteins (Gemma et al, 2007).

A large body of studies supports oxidative damage as contributing factor to development of AD and may be as one of the earliest features in the onset of the disease. MCI maybe considered as one of the earliest phases in the development of AD because most people with mild cognitive impairment (MCI) ultimately progress to AD (Keller et al, 2005).

Antioxidant vitamins, including vitamins E, the most potent lipophilic chain-breaking antioxidant, (Stocker R, 2007) and Vitamin C, a potent water soluble antioxidant, may reduce neuronal damage and death from oxidative reactions by inhibiting the
production of ROS, lipid peroxidation, apoptosis, protein oxidation, as well as damage to cell membranes and/or DNA (Maxwell et al, 2005).

Bioavailability, metabolism and plasma level of vitamin E have been studied in healthy humans and in AD patients (Polidori et al, 2007; Iuliano et al, 2010).

Many Studies show that vitamin E is able to cross through the blood brain barrier and to accumulate at treatment levels in the central nervous system (CNS). It also counteracts neuronal damage in the brain by reducing isoprostane levels (Praticò et al, 2002) and it is able to decrease lipid peroxidation in CNS (Sung et al, 2004). ROS is neutralized by vitamin E and may be especially important in protecting lipid membranes in the oxygen rich environment of the brain.

Animal models indicate that 40% of vitamin C content in the brain turns over daily. A concentration of 10 μM which is able to reduce amyloid-β and regenerate other antioxidants will be maintained in neurons (Bowman et al, 2009).

Effects of vitamin E and C in combination with diets rich in these vitamins have shown a protective effect against AD. It is very likely that a single antioxidant cannot act as a neuroprotection in MCI because there is widespread oxidation of multiple macromolecules of lipids, proteins, DNA, and RNA in this disorder. Combined antioxidants will be needed to up-regulate defense mechanisms against oxidation and to neutralize the oxidative component of the pathogenesis of AD (Markesbery and Lovell, 2007).

Vitamin E becomes oxidized in the process of neutralizing ROS, losing its oxidative ability. Vitamin C readily donates electrons to vitamin E to restore its oxidative ability (Wengreen et al, 2007).

The aim of the present study is to examine whether antioxidant supplements, vitamin E and C, have actually any significant effect on cognitive performance of a group of elderly males and females in Iran.
2. GOAL AND OBJECTIVES

2.1. Goal

The goal of this study is to investigate the effect of an intervention with antioxidant supplements vitamin E and C on elderly people suffering from Mild Cognitive Impairment (MCI) in Isfahan, Iran

2.2. Analytical objectives

**Related to score of cognitive function:**

1. To compare the mean score of cognitive function assessed by Mini Mental State Examination questionnaire at baseline, six\textsuperscript{th} and twelfth months after intervention \textit{between} supplemented and control group and three age subpopulations

2. To compare the mean score of cognitive function assessed by Mini Mental State Examination questionnaire at baseline, sixth and twelfth months after intervention \textit{within} supplemented and control group

3. To compare the mean score of cognitive function assessed by Mini Mental State Examination questionnaire at baseline, sixth and twelfth months after intervention among three age subpopulation

**Related to serum redox status:**

1. To compare serum redox status assessed by MDA, GSH, TAC and 8-OHdG at baseline, sixth and twelfth months after intervention \textit{between} supplemented and control group and three age subpopulations.

2. To compare serum redox status assessed by MDA, GSH, TAC and 8-OHdG at baseline, sixth and twelfth months after intervention \textit{within} supplemented and control group
3. To compare serum redox status assessed by MDA, GSH, TAC and 8-OHdG at baseline, sixth and twelfth months after intervention among three age subpopulation.

**Related to dietary intake parameters:**

1. To compare dietary intakes of vitamin E, vitamin C, total carotene, zinc and selenium assessed by 3-day food records at baseline, sixth and twelfth months after intervention between supplemented and control group

2. To compare dietary intakes of vitamin E, vitamin C, total carotene, zinc and selenium assessed by 3-day food records at baseline, sixth and twelfth months after intervention among three age subpopulations

**Individual related parameters:**

1. To compare the body mass index at baseline between supplemented and control group and three age subpopulations

2. To compare the maximum and minimum blood pressure at baseline between supplemented and control group and three age subpopulations

3. To compare other confounding factors at baseline between supplemented and control group and three age subpopulations

**2.3. Hypothesis of the survey**

1- There are improvements in cognitive function after supplementation of vitamin E and C in supplemented group compared with the control group.

2- There are improvements in redox status after dietary supplementation of vitamin E and C in supplemented group compared with the control group.
3. LITERATURE REVIEW

3.1. Mild Cognitive Impairment

3.1.1. Concept of Mild Cognitive Impairment (MCI)

Mild cognitive impairment (MCI) was first defined in 1999 as a pathological brain condition. Epidemiological studies of aging and dementia have shown that the classification of dementia identifies three groups of subjects: those who are affected by dementia, those who are not demented, and individuals who have cognitive (memory) impairment but do not meet criteria for dementia (Petersen et al, 2001).

The concept of MCI was proposed to describe this transitional zone between normal aging and dementia (Huang C, 2003).

MCI is characterized by a measurable memory impairment that is abnormal for an individual’s age and education, and is confirmed by an informant (Petersen RC, 2000). It is estimated with a probability of 50% within 4 years, approximately 12% per year, the progression of MCI into Alzheimer’s disease. It’s supporting the concept that MCI represents a prodromal stage of AD (Baldeiras et al, 2008), although ~5% of MCI patients remain stable or revert to normal (Lovell and Markesbery, 2007).

3.1.2. Criteria for the MCI

1. Concern about alteration in cognition

There should be evidence of concern about cognition change, in comparison with the person’s prior level. This concern can be obtained from the patients who suffering from MCI, informant or caregiver who knows the patient well, or from a skilled clinician observing the patient.

2. Impairment in one or more cognitive domains

People with MCI are usually facing to lower performance in one or more cognitive domains. This change can occur in different domains of cognitive including: memory
(i.e., the ability to learn and retain new information), executive function, attention, language and visuospatial skills. This problem is greater than expected for the patient’s age and educational level.

3. Preservation of independence in functional abilities

People with MCI are usually able to perform complex functional tasks such as shopping, paying bills and preparing a meal with mild problem. They make more errors to carry out such activities than in the past. Nevertheless, they try to be independent in their daily life, with minimal aids or assistance (Albert et al, 2011).

4. Not demented

MCI is characterized with a mild deficit in memory or some aspect of cognitive domain without dementia. In fact, the patient with mild cognitive impairment is neither normal nor demented (Petersen et al, 2001).

3.1.3. Classification of MCI

Mild cognitive impairment can be classified to two subtypes including amnestic (having memory deficits) or non-amnestic. They were then further divided to single or multiple cognitive domains (figure 1). For instance, a patient whose only cognitive complaint is memory deficits would be categorized as having “amnestic MCI, single domain (A-MCI).” A patient with memory deficits in addition to difficulties in non-memory cognitive domains such as problem solving or language would be categorized to have “amnestic MCI, multiple domains.” The patient would be categorized as having “non-amnestic MCI,” while there are complaints in non-memory cognitive domains. It could similarly be single or multiple domains (Rosenberg et al, 2006). Amnestic MCI may act as the earliest point at which treatments for dementia, particularly AD, can be attempted (Petersen RC, 2000).
Figure 1. Flow diagram for diagnosing MCI subtype classification

(Petersen and Negash, 2008).
3.1.4. Diagnosis of MCI

MCI can be diagnosed when cognitive decline is not severe enough to affect daily living, but sufficient to be annoying and measurable by psychometric and other clinical assessments (Kidd PM, 2008).

Patients who do not have dementia and are abnormal for an individual’s age and education remain diagnostic challenges. In fact, the concept of normality in aging is itself controversial. Although, most elderly people report that their memory and cognition are worse than it was when they were younger. The majority of experts believe that normality must be determined with respect to a particular age group (Chertkow et al, 2008).

MCI can be currently diagnosed by the informant and judgment of a clinician and there is not any laboratory test for identifying it. Thus, MCI is a syndrome defined by clinical, cognitive, and functional criteria.

Other cognitive domains among people with MCI can be affected and should be examined in addition to memory. These include: executive functions (reasoning, problem-solving, planning), language (naming, fluency, expressive speech, and comprehension), visuospatial skills, and attentional control (e.g., simple and divided attention) (Albert et al, 2011).

Objective report of decline in memory and learning on brief or extensive cognitive testing and subjective report of cognitive decline from a previous level, for at least 6 months are needed for initial criteria for MCI.

In all cases, the people who suffering from mental illnesses such as significant depression, delirium, mental retardation or other psychiatric disorders that are likely responsible for the impairment are excluded.

The patients with the severe memory loss along with significant functional disorder and other cognitive impairments indicate the clinical criteria for dementia, not mild cognitive impairment (Chertkow et al, 2008).

A new diagnostic procedure on MCI with three separate parts has been suggested by the European Consortium on Alzheimer’s Disease Working Group. The consortium emphasizes that different clinicians should be participated at each stage.
The first stage of the diagnostic procedure is detection of Mild cognitive impairment. MCI should correspond to cognitive complaints coming from the patients or informant during the past year. Cognitive disorders in memory and other cognitive domain have to be evidenced by clinical evaluation. Secondly, subtypes of MCI syndrome have to be recognized. Finally, the underlying aetiopathogenic subtype could be identified (Portet et al, 2006).

For the determination the accuracy of the MCI diagnosis, objective report of progressive impairment in cognition is very important over time (Figure 2) (Chertkow et al, 2008).

Previous studies have found that there are a variety of episodic memory tests that are applicable for evaluation of MCI patients. These tests assess both immediate and delayed recall, in order to determine retention over a delay. Most of the tests which are useful in this regard are wordlist learning tests with multiple trials. Such tests indicate the rate of learning over time, as well as the maximum amount acquired over the course of the learning trials (Albert et al, 2011). These tests have been widely described as good predictors for AD in MCI subjects. MMSE (Mini-Mental State Examination) is currently used tests for screening of cognitive impairment throughout the world, and it has been reported that decline in MMSE score starts approximately three years before the diagnosis of dementia (Pozueta et al, 2011).
Figure 2. Flowchart for differential diagnosis of MCI

(Rosenberg et al, 2006)

3.1.5. Treatment of MCI

Reports regarding the treatment of MCI have either been symptomatic, usually of shorter term, or of longer multiyear period to demonstrate whether disease progression is delayed. There were no symptomatic drug study to show clinically convincing differences between placebo and the study medication (Farlow, 2009). In some randomized
clinical trials, cholinesterase inhibitors and vitamin E have failed to prevent progression of mild cognitive impairment to dementia while some others yielded positive results. For example, Petresen et al in their randomized clinical trial study indicated that Donepezil as cholinesterase inhibitors was found to have transient preventive effect at 1 year, with a larger and more sustained effect in subjects who had at least one apoE4 allele (Petersen et al, 2005). Few studies have examined the relation between dietary intake of antioxidant nutrients and the development of AD (Morris et al, 2002). Some prospective studies have found less cognitive decline with the use of vitamins C and E than without their use (Masaki et al, 2000; Paleologos et al, 1998; Morris et al, 1998) whereas others found no reduction in incident cognitive impairment or Alzheimer’s disease with the use of vitamin C, vitamin E, or beta-carotene supplements (Morris et al, 1998; Engelhar et al, 2002).

3.2. Alzheimer’s disease (AD)
AD was first identified by Alois Alzheimer, a German psychiatrist and neuropathologist. He explained the clinical and pathological findings of a case study (51-year-old woman) with a 4.5 year course of progressive dementia which is subsequently recognized as a disorder bearing his name (Alzheimer A, 1907). AD is the fourth leading cause of death in the United States. The number of Americans with Alzheimer’s disease and other dementias will grow each year and lack of preventive strategies, there may be 14 million Americans with AD by the year 2040 (Hebert et al, 2003).

Autopsy of this patient revealed the presence of silver positive neurofibrillary tangles (NFT) and severe cerebral cortical neuron loss which is subsequently known as senile plaques (SP) (Markesbery, 1997). Research into its symptoms, causes, risk factors and treatment has gained momentum only in the last 30 years.
3.2.1. Symptoms of Alzheimer’s disease

Alzheimer’s disease as a progressive illness affects people in different ways but the most common early symptom of Alzheimer’s disease is difficulty remembering newly learned information. This occurs in brain regions involved in forming new memories due to disruption of brain cell function. Signs of Alzheimer’s are as follows:

- Memory loss that impair daily living
- Having trouble with planning or solving problems
- Disability in familiar tasks at home, at work or at leisure
- Confusion with time or place
- Having trouble in visuospatial functions
- Changes in processing speed, attention, speaking or writing, judgment, mood and personality
- Misplacing things and losing the ability to retrace steps (Alzheimer’s Association, 2012).

The cause(s) of Alzheimer’s disease are not yet known. An excessive production and accumulation of amyloid-β (Aβ) in senile plaques may be a critical factor for the onset of the disease, although the mechanism by which Aβ causes damage and eventually neuronal death is still unknown (Mattson PM, 2004).

Clinically, AD is marked by a progressive decline in multiple cognitive functions and is thought to begin with MCI (Morris et al, 1989).

Numerous experiments have revealed that oxidative damage may play an important role in the pathogenesis of neurodegenerative disease, including AD (Solfrizzi et al, 2006), and it can act early during the disease, preceding the development of the pathological hallmarks, like neurofibrillary tangles and senile plaques (Nunomura et al, 2001).

3.3. Oxidative Stress

Oxidative stress (OS) has been defined as an imbalance between cellular production of ROS and the inability of cells to defend against them (Gilgun-Sherki et al, 2001). In fact, when highly reactive free radicals activity exceeds the antioxidant systems
defense and not tightly kept under control, it can potentially damage all components of the cell (Preiser JC, 2012). The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process (Dröge W, 2002).

3.3.1. Reactive free radical
Free radicals are defined as molecules or molecular fragments having one or more unpaired electrons in the outer shell and generally unstable and very reactive (Halliwell and Gutteridge, 1999). The detrimental effect of free radicals is oxidative stress and nitrosative stress (Kovacic and Jacintho, 2001; Ridnour et al, 2005; Valko et al, 2001). Reactive oxygen species (ROS) and reactive nitrogen species (RNS), include superoxide, hydroxyl, peroxyl (RO2•), alkoxy (RO•), and hydroperoxyl (HO2•) radicals (Evans and Halliwell, 2001; Jung et al, 2009). Electromagnetic radiations, medication, man-made pollutants as well as cellular metabolisms constantly induce free radicals in living organism. Overproduction of these highly reactive species or deficiency of enzymatic and non-enzymatic antioxidant defense could lead to oxidative stress (Gilgun-Sherki et al, 2003).

3.3.2. Production of Reactive Oxygen Species (ROS)
When oxygen as a vital element is used by cells to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. These by-products are generally ROS as well as RNS that are created by the cellular redox process (reduction and oxidation). They play dual role and can be either harmful or helpful to the body. ROS and RNS in low or moderate concentrations have beneficial effects on cellular responses and immune function but in a high level, they generate oxidative stress that can damage cell structures, including lipids, proteins, and DNA (Halliwell and Gutteridge, 2007; Young and Woodside, 2001). Mechanisms of production of ROS are shown in figure 3.
Superoxide (O2•) is generated from O2 by oxidases such as NADPH-oxidase. One electron from a donor molecule is transferred to oxygen by this oxidase and produce superoxide (O2•). From superoxide, hydrogen peroxide (H2O2) is created by “dismutation” (spontaneous or enzymatic). H2O2 is relatively stable and can be diffused far from its production site. There are possible subsequent steps include (1) convert to H2O by the glutathione peroxidases or catalase and/or by the mitochondrial respiratory chain complexes, (2) creation of hydroxyl (•OH) in the presence of iron (eg, ferrous in heme groups), or (3) formation of hypochlorous acid (HOCl) as a powerful oxidant by peroxidases, with a lifetime sufficient to diffuse in and out of the cell. It will be able to oxidize almost all types of biomolecules. There is a close connections between the metabolism of ROS and the metabolism of RNS, mainly nitric oxide (NO•). NO• as a free radical is formed by L-arginine, able to diffuse freely, and its activity is limited by its short lifetime. Peroxynitrite (ONOO−) whose oxidant potential is greater than that of O2• or H2O2 alone is the most toxic derivate of NO•. As shown in figure3, ONOO− results from the combination of NO• with superoxide can be highly cytotoxic. Due to instability of Peroxynitrite, it can be converted into new active species, such as hydroxyl radical (•OH) and nitryl radical (•NO2). They are responsible for hydroxylation and nitration respectively. NO• can also react with O2 to generate nitrite (NO2−) in appropriate conditions (Preiser JC, 2012).
3.3.3. Modulating and Stopping the Oxidative Chain

Oxidation as a natural process inducing damage to all living cells found in nature. Antioxidants are able to counteract the harmful effects of oxidation in body tissues (Kuhn MA, 2003; Goodyear-Bruch and Pierce, 2002).

A series of defense mechanisms are developed when the body exposed to a variety of free radicals (Cadenas E, 1997).

The human body has several defense mechanisms against free radical-induced oxidative stress include preventative mechanisms, repair mechanisms, physical defenses, and antioxidant defenses.
Enzymatic antioxidant defenses are represented by superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT).

Non-enzymatic antioxidants include vitamin C (ascorbic acid), vitamin E (α-tocopherol), glutathione (GSH), carotenoids, flavonoids, and other antioxidants (Valko et al, 2007).

The antioxidant systems are divided to stoichiometric (scavengers of ROS) and catalytic (detoxifying enzymatic systems) functionally (Preiser JC, 2012).

3.4. Non-enzymatic antioxidants

3.4.1. Vitamin E (D-α-tocopherol)

Vitamin E, the most potent lipophilic chain-breaking antioxidant is an essential nutrient in humans. It plays an important role in scavenging free radicals in cell membranes, inhibits ROS-induced generation of lipid peroxyl radicals and β-amyloid deposition in the brain by reducing isoprostanone levels (Sung et al, 2004; Praticò et al, 2002; Meydani M, 1995). Vitamin E has eight stereoisomers including: alpha, beta, gamma, delta tocopherol and alpha, beta, gamma, delta tocotrienol. Only alpha-tocopherol is the most biologically form in humans (Pham-Huy et al, 2008). Very common is the esterified form Dl-α-tocopherol acetate. The biological activity of tocopherols and tocoterinols is lower than that of Dl-α-tocopherol acetate. 1mg D-α-tocopherol =1.49 mg Dl-α-tocopherol acetate= 1.5 IU.

This vitamin is able to cross the blood brain barrier and to accumulate at therapeutic levels in the central nervous system. Vitamin E has been proposed for the prevention against colon, prostate and breast cancers, some cardiovascular diseases and certain neurological disorders (Pham-Huy et al, 2008). Much of the available clinical and epidemiological studies have indicated that vitamin E can delay the onset of cognitive decline (Grodstein et al, 2003; Engelhart et al, 2002). Vitamin E are abundant in vegetable and wheat germ oil, whole grains, nuts, cereals, fruits, eggs, poultry, meat and etc. (Willcox et al, 2004). Cooking and storage may destroy natural D-alpha-tocopherol in foods (Pham-Huy et al, 2008).
3.4.1.1. Dosing of vitamin E (D-α-tocopherol)

The Recommended Daily Allowance (RDA) of vitamin E (D-α-tocopherol) for adults is 15mg (or 22.5 IU) and for supplementary using, the tolerable upper limit (UL) recommended by the U.S. Institute of Medicine is 1000mg/day (equivalent to 1500 IU) (Miller et al, 2005). According to recommendation of European Food Safety Authority (EFSA), the UL for vitamin E (D-α-tocopherol) was established as 270 mg/day for adults and rounded to 300 mg/day. However, based on a recent trial, daily doses of 400 IU or more alpha-tocopherol can increase the risk of death and should be avoided. In contrast, there may even be some advantage with a dose of 200 IU per day or less (Miller et al, 2005; Barbul and Uliyargoli, 2007). Higher dose and long-term of vitamin E supplementation should be used carefully until further evidence for its safety is available (Miller et al, 2005). Normal plasma concentrations of vitamin E in humans range from 11.6 to 30.8 μmol/L (Meydani M, 1995).

3.4.2. Vitamin C (Ascorbic acid)

A potent water-soluble antioxidant is known as ascorbic acid. It is essential for plasma (Frei et al, 1989) and in the cerebrospinal fluid (CSF) (Arlt et al, 2000). It also needed for collagen, carnitine and neurotransmitters biosynthesis.

Saturable sodium-dependent vitamin C transporter (SVCT1) carries the ingested vitamin C from the intestines into blood and brain (Harrison FE, 2012).

It is estimated that there is between 100 and 500 μmol/L of vitamin C concentrated in the brain. Although, due to neural activity, concentration of vitamin C in the brain changes rapidly, its physiologic functions are not well understood (Grunewald, 1993). Animal models have shown that approximately 40% of Ascorbic acid (AA) content in the brain turn over daily and it maintenance as high as 10 μM in neurons (Rice, 2000). This concentration of AA is able to reduce oxidative neuronal stress through protection of cell membranes and DNA from oxidation, reducing amyloid-β toxicity and regenerating other antioxidants (Floyd and Hensley, 2002; Drew et al, 2002).
In humans, most plasma components are diluted about 100-fold in cerebrospinal fluid (CSF) but vitamin C in CSF is highly concentrated compared to plasma (Quinn et al, 2003); supporting statements by some investigators that AA surrounds the brain constantly (Davson and Segal, 1996). The advantages of vitamin C include antioxidant, anti-atherogenic, anti-carcinogenic and immune-modulator. Vitamin C are abundant in citrus fruits, pineapple, strawberry, green vegetables, tomatoes, etc. Ascorbic acid is an unstable molecule and may be lost during cooking (Dhanasekaran and Ran, 2005; Maharaj et al, 2007).

3.4.2.1. Dosing of Vitamin C

The recommended daily allowance (RDA) for ascorbic acid ranges between 100–120 mg daily for adults. However, the intake of higher dose of vitamin C (2000mg or more/day) is controversial for its eventual pro-oxidant or carcinogen property (Dhanasekaran and Ran, 2005; Maharaj et al, 2007).

High intakes of vitamin C may result in diarrhea and abdominal bloating for some people, and is not suitable for patients who have iron overload, hemochromatosis, thalassemia major or other diseases requiring multiple red blood cell transfusions (Cohen et al, 1981).

3.4.3. Synergistic effects of vitamin C and E

Synergistic antioxidant functions of vitamin C and vitamin E are supported by some of the biological evidences.

Vitamin E donates electrons to counteract ROS and may be especially important to protect lipid membranes in the oxygen rich environment of the brain. Vitamin E becomes oxidized in the process of neutralizing free radicals, losing its oxidative ability; vitamin C, as shown in figure 4 gives electrons to vitamin E to restore its oxidative ability (Zandi et al, 2004).
The protective effect of ascorbic acid alone was negligible compared with the effect of an equimolar concentration of α-tocopherol. However, Yeum et al have also indicated that combined of these two vitamins have protective effect compared with single antioxidant (Yeum et al, 2009). A previous study based on supplements has provided evidence for the use of vitamin E and C supplements in combination. They indicated that such combination was associated with reduced prevalence and incidence of Alzheimer’s disease (Zandi et al, 2004).

![Figure 4. Pathways of ROS formation, the lipid peroxidation process, role of GSH and other antioxidants](Valko et al, 2007)

The vitamin E radical (T-O) is reduced back to vitamin E (T-OH) by ascorbic acid (the physiological form of Ascorbate is Ascorbate monoanion, AscH⁻) leaving behind the ascorbyl radical (Asc⁻). The oxidized vitamin E radical (T-O) is also reduced by GSH (Reactions of 8, 9 and 10).
3.5. Cognitive Impairment and Antioxidants

3.5.1. Animal Studies

Most studies in the field of animal cognition have provided information regarding the basic mechanisms of cognition that may not be possible to obtain from humans due to several limitations and variations.

In order to determine whether short-term vitamin E supplementation could reverse age-associated impairments in cognitive or motor function, sixty young and sixty old male mice were selected. Mice were placed on either a control diet or the same diet supplemented with 1.65g/kg α-tocopheryl acetate. After 4 weeks of supplementation and continued for the subsequent 8 weeks, a swim maze test was used to assess cognitive and psychomotor performance.

The concentration of α-tocopherol in the cerebral cortex (Site for learning) among both young and old mice was increased in vitamin E supplementation but did not significantly influence on oxidative damage to proteins and lipids in the brain cortex. Also, in spite of the mice had been supplemented for up to 10 weeks prior to testing, this supplementation did not significantly influence on cognitive performance in terms of several tests for motor skills and sensory reactivity (Sumien et al, 2004).

In another study, the relation between use of vitamin E + C and cognitive performance was examined among two groups of young and aged mice. Each group was following divided into four groups (n = 10). All drugs were administered once a day orally for 2 months. The first and second groups received vitamin C alone (300 mg/kg) and vitamin E alone (250 mg/kg) respectively. The third group received combined vitamin C (300 mg/kg) and vitamin E (250 mg/kg) and the fourth group received normal saline (1 ml/100 g). Recording of memory indices began 24 h after administration of the last dose of drugs. The result revealed no significant effect of vitamin E supplementation on memory in young mice. The chronic administration of vitamin E alone or
combined vitamin E and vitamin C caused significant improvement in the memory of aged mice. Administration of vitamin C alone among aged mice compared to the control group did not show any significant difference (Arzi et al, 2004).

Another study focused on effects of vitamin E and the cholinergic system on memory retention of passive avoidance learning in rats. Eight Male Wistar rats, weighing 200-250 g were used in each experiment. The drugs included vitamin E acetate (10, 25 and 50 microg/rat) and/ or nicotine, pilocarpine, muscarinic, mecamylamine and scopolamine. Intra-cerebroventricular (I.C.V.) injections were carried out in all experiments. The result indicated that vitamin E attenuated mecamylamine or scopolamine-induced impairment on memory retention. In addition, vitamin E increased strength of nicotine or pilocarpine (agonist increased memory retention); indicating that vitamin E and cholinergic system have a close interaction. This report suggested that vitamin E may act through activation of cholinergic system on memory retention and affects sensitivity of cholinceptors in the brain. Therefore, it has a close interaction with the cholinergic system on memory retention process (Eidi et al, 2006).

In order to assess the susceptibility against oxidative stress, three groups of rat were selected as follows: young rats aged 3 months, old rats aged 25 months and vitamin E–deficient rats. All animals were tested for learning and memory. For exposing to oxidative stress, young animals were kept under oxygen 100% at 20°C for 48 hours. Young rats showed significantly greater learning ability before the stress than the old and vitamin E–deficient rats. At five days after stress, the memory performance of the young rat was declined toward the level of that in the aged rats maintained under normal condition. After vitamin E supplementation, learning performance of young rat increased significantly before the stress and prevented the deficit of memory caused by the stress. These results suggest that chronic oxidative stress may contribute to the deficit of the learning and memory function during aging, because of the neuronal apoptosis of the
cerebral cortex (site for learning) and hippocampus (site for memory) and vitamin E can accelerated significantly their learning functions before exposing to stress (Fukui et al, 2002).

Another study was divided to two studies. In the first study, 12 mice aged 5 months were divided to two groups. They randomized to receive a diet supplemented with vitamin E (2 IU/g diet) or placebo until they were 13 months old, and then killed. In the second study, 12 mice aged 14 months were divided into two groups. They also randomized to receive the same diet supplemented with vitamin E or placebo until they were 20 months old, and then killed. Level of their plasma vitamin E was increased in the end of study among supplemented group. The administration of vitamin E in two groups significantly reduced 8, 12-iso-iPF2α-VI levels (marker of in vivo lipid peroxidation) in cortex as well as in hippocampus homogenates, compared with placebo. This reduction inversely correlated with a significant increase of vitamin E levels in the same brain regions. Vitamin E also significantly reduced Aβ (β-amyloid) levels and amyloid plaque deposition in mice when it is administered early during the evolution of their disease phenotype. By contrast, if vitamin E supplementation is started at a later age, when amyloid plaques are already deposited, despite a reduction in brain oxidative stress, no significant effect is observed on the amyloidotic (deposition of amyloid in an organ) phenotype of these animals (Sung et al, 2004).

3.5.2. Human Studies

Information concerning the advantages of supplemental antioxidant use for the prevention or delay the onset of cognitive decline in healthy individuals has yielded conflicting results. Some epidemiological and clinical studies have investigated less cognitive decline with the use of vitamins E and C than without their use, while others indicated no reduction in incident cognitive impairment or Alzheimer’s disease with the use of vitamin E, vitamin C, or beta-carotene supplements.
For the first time, Morris et al have examined the association between antioxidant supplements vitamin E & C and the prevention of Alzheimer disease in a prospective study of 633 healthy persons 65 years and older. Vitamin supplement use was classified to three levels of intake: subjects who used specific vitamin supplement, second group including subjects who used only a multivitamin supplement containing the vitamin and the third group was nonuse of any supplement containing the vitamin. After an average follow-up period of 4.3 years, 91 of the participants were diagnosed with AD, but none of the 27 vitamin E supplement users and 23 vitamin C supplement users had AD. In this study, there was no relation between Alzheimer disease and use of multivitamins (Morris et al, 1998).

Another double-blind, placebo controlled trial on 341 patients suffering from moderate to severe Alzheimer’s disease was carried out. Duration of supplementation was 2 years. Vitamin E (racemic mixture of dl-alpha-tocopherol) (2000 IU/d or 1300mg) and/or selegiline (a monoamine oxidase inhibitor) have been tested in this study. There were significant delays in the time of death and disability for the patients treated with selegiline, vitamin E, or combination therapy as compared with the placebo group while investigators didn’t observe any improvement in cognitive test scores in the treatment group. MMSE questionnaire was used in their trial (Sano et al, 1997).

Anthony et al conducted a cross-sectional survey using 4,809 sample related to three groups including: non-Hispanic White, non-Hispanic Black, and Mexican-American elderly. They found out that increasing levels of vitamin E were associated with better memory function for this ethnically diverse elderly population. Low levels of vitamin E per unit of cholesterol were associated with poor memory among all three race-ethnicities. However, no relations of vitamin A, β-carotene, selenium and vitamin C, any of the other antioxidants or essential trace elements with poor memory were indicated (Anthony et al, 1999).
In another study, subjects were a group of 120 elderly men and women who were free of significant cognitive impairment. Serum levels of α-tocopherol and cholesterol were measured and dietary intake using a “weighed food record” for 5 successive days was determined. The cognitive performance of subjects using the Pfeiffer’s Mental Status Questionnaire (PMSQ) was evaluated. Subjects with vitamin intakes lower than 50% of the RDI (Recommended Daily Intake) made more errors on the PMSQ test than participants with a higher intake of the vitamin. Subjects who made no errors on the PMSQ test had significantly higher serum level of α-tocopherol and α-tocopherol/cholesterol ratios than those who made errors (Ortega et al, 2002).

Petersen et al conducted a randomized, double-blind, placebo-controlled study. Subjects with amnestic MCI were randomly assigned to receive 2000 IU (1300 mg) of vitamin E or 10 mg of donepezil (the most widely used cholinesterase inhibitor) daily or placebo for three years. Progression to AD by standard diagnostic criteria was the primary outcome. A total of 769 patients were enrolled but 239 dropped out during the follow-up phase. At the end of the study, 212 developed AD, with 16% conversion rate per year. There were no significant differences in the rate of development to AD between vitamin E and placebo groups at any points. In addition to progression to AD, they also noticed the changes of cognition, assessed by several tools including MMSE. This study indicated that vitamin E, even when taken in large doses over long periods of time, is not able to improve or maintaining the MCI. (Petersen et al, 2005).

Another prospective study namely, the Cache County Study, revealed no remarkable reduction in risk of incident AD with vitamin E or vitamin C or multivitamin in 4740 subjects. This study has also shown use of combined vitamin E and C supplements was associated with reduced AD prevalence and incidence (Zandi et al, 2004).

In another clinical trial study, a group of 57 patients with AD and 18 healthy controls were enrolled. They were randomly assigned to receive vitamin E (800 IU per day or 530 mg), or placebo for six months. Blood total glutathione levels, oxidized
glutathione (GSSG) and plasma malondialdehyde were determined. Cognitive performance was assessed using three tools including the Mini-Mental State Examination (MMSE), Blessed-Dementia Scale and Clock Drawing Test at baseline and post intervention. In some cases, vitamin E treatment resulted in a reduction of the GSSG levels while in other patients did not respond to vitamin E treatment. The non-respondent group of patients did not ameliorate their cognitive performance when treated with vitamin E. In fact, in some of the cases, vitamin E treatment was even worse than placebo group. The results showed that giving vitamin E to AD patients may be detrimental, especially if the oxidative stress status of the patients is not carefully monitored (Lloret et al, 2009).

In order to determine the effect of antioxidants or zinc and copper on cognitive performance among 2166 participants, a large clinical trial as part of AREDS (Age-Related Eye Disease) was conducted. Subjects had a mean age of 75± 5 years for an average of nearly 7 years. The cognitive performance battery included six validated cognitive tests. Participants were randomly assigned to receive daily oral tablets containing 1) antioxidants (500 mg vitamin C, 400 IU vitamin E or 260 mg, and 15 mg beta carotene; 2) 80 mg zinc as zinc oxide and 2 mg copper as cupric oxide; 3) antioxidants plus zinc and copper; or 4) placebo. Supplemented group with or without zinc and copper compared with placebo did not have a significant effect on cognitive function at the end of the trial. There were also no significant differences in the likelihood of having cognitive impairment, as assessed by the Modified Mini-Mental State (3MS), across all treatment groups (Yaffe et al, 2004).

A prospective study to examine the effect of combined vitamin E and C with NSAIDs (non-steroidal anti-inflammatory drugs) on cognitive performance in a group of 3376 elderly was carried out. Cognitive function by Modified Mini-Mental State (3MS) up to three times over 8 years was assessed. Investigators found an association between using a combination of vitamins E and C supplements plus NSAIDs and less cognitive
decline among elderly APOE ε4 allele (Apolipoprotein E) carriers. In this study, taking antioxidant supplements and NSAIDs together was especially advantageous and had noticeable effects on 3MS trajectories even without consideration of the timing of their use (Fotuhi et al, 2008).

In another large cohort of community-dwelling women, information regarding supplement use containing vitamin E and C via mailed questionnaires has collected. A battery of cognitive tests to 14968 of the elderly individuals by telephone interview for evaluation cognitive performance which is modeled on the Mini-Mental State Examination was administered. The results revealed better overall performance on cognitive tests among long-term users of combined vitamins E and C than among women who had never taken either vitamin. Investigators also showed little support for vitamin C and vitamin E supplements alone on cognitive function and there was no evidence of a trend with duration of use. This report also provides evidence that increasing duration of the supplementation period improved likelihood of appearance of their beneficial effect on cognition (Grodstein et al, 2003).

A total of 84 elderly aged between 60 and 74 years participated in a cross-sectional study to examine the effect of dietary antioxidant intake on MCI. Data on food intake was assessed using the diet history and food frequency questionnaire. A combination of tests including: MMSE, Clock Drawing Test (CDT), and Dementia Rating Scale (DRS) to evaluate cognitive function were used. The average intake of vitamin C and beta-carotene were satisfactory but the average vitamin E intake among subjects did not meet the RNI (Recommended Nutrient Intakes). After adjusting for education, a weak but significant association was found between beta-carotene intake and cognitive performance in CDT (Wong et al, 2010).
In order to examine the effect of the antioxidant flavonoid Pycnogenol on a range of cognitive and biochemical measures in healthy elderly individuals, a double-blind, placebo-controlled, matched-pair study was conducted. A group of 101 elderly participants aged between 60-85 years consumed a daily dose of 150 mg of Pycnogenol for a three-month treatment period. Participants in four time points including: at baseline, then at 1, 2, and 3 months of the treatment were assessed. The Clinical Dementia Rating (CDR) was used to evaluate a range of cognitive performance including attention, information processing, memory function, reasoning, secondary memory, and skilled coordination.

Compared with the control group, supplemented group showed the improvement in quality of working memory factor following intake of Pycnogenol from second to third months of treatment. Post hoc comparisons revealed no differences between the groups at baseline, 1 month, or 2 months (Ryan et al, 2008).

In order to evaluate the relationship between supplemental use of antioxidant vitamins and subsequent risk of cognitive decline, a 5 year prospective population-based study was conducted. A total of 10263 subjects of Canadians elderly aged 65 years and older participated in this study. Information regarding vitamin E or C supplement use alone and for the combined use of vitamin E and C and/or multivitamins was collected. Dosage and duration of supplement use was not available. Primary outcome of significant cognitive decline was defined as a decrease in Modified Mini-Mental State (3MS) score of 10 points or more between baseline assessment and second cycle of the study. The results showed that subjects using both vitamin E and C supplements and/or multivitamins were significantly less likely to experience a cognitive decline during the 5-year follow-up period. Also, older participants who reported supplemental use of any antioxidant vitamin at baseline (E, C or multivitamin) were significantly less likely to experience significant cognitive decline (Maxwell et al, 2005).
A placebo controlled, double-blind trial using 600 IU of α-tocopherol every other day starting 5.6 years after initiation of the WHS (The Women's Health Study) and running for four years' duration (totally 9.6 years). The initial trial enrolled 6377 women, of whom 5,073 had mental status.

Researchers found no differences in global score between supplemented and placebo groups at the first assessment (5.6 years after randomization) or at the last assessment (9.6 years of treatment). Mean cognitive change over time was also the same in the vitamin E group compared with the placebo group for the global score. The relative risk of substantial decline in the global score in the vitamin E group compared with the placebo group was 0.92. In conclusion, this report provides evidence that among more than 6000 healthy women, vitamin E supplementation did not provide overall cognitive benefits or reduce cognitive decline (Kang et al, 2006).

Effect of vitamin E and C supplementation on lipoprotein oxidation in patients with Alzheimer's disease was determined. The trial revealed that the supplementation of 20 AD patients with 400 IU vitamin E and 1000 mg vitamin C in combination for 1 month significantly increased the concentrations of both vitamins in CSF and plasma. In contrast, the one month vitamin E supplementation with 400 IU significantly increased its concentrations in CSF and plasma while it was unable to decrease the lipoprotein oxidizability (Kontush et al, 2001).

In another study, the enzymatic activities of the erythrocyte Cu-Zn superoxide dismutase (Cu-Zn SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and plasma level of total antioxidant status (TAS) in AD and control groups were assessed. A group of 91 Iranian with AD with mean age of 75 ±10 years and 91 controls were selected. In this case-control study, the activity of erythrocyte Cu-Zn SOD in AD patients compared with age-matched control was increased considerably. The mean plasma concentration of TAS and the activity of erythrocyte GSH-Px were lower in AD patients than in control group. These results confirm the idea that the oxidative
stress plays a major role in the pathogenesis underlying AD neuro-degeneration (Vaisi-Raygani et al, 2008).

3.6. Mechanism of action:

3.6.1. Introduction

Alzheimer’s disease (AD) is the most known cause of dementia in the elderly, characterized by severe cognitive and functional disabilities and may have a long stage of neuropathological changes before it is diagnosed (Clark CM, 2000). Several lines of research clearly indicate that the onset of AD is typically preceded by a transitory state known as mild cognitive impairment (MCI) (Flicker et al, 1991; Petersen et al, 1995). In its amnestic version, as a prodromal stage of AD, MCI is marked primarily by a memory loss without clinically meaningful functional disability and is abnormal for an individual’s age and education and is confirmed by an informant (Tuokko and Frerichs, 2000; Petersen RC, 2000; Petersen et al, 1999).

According to the free radical theory, aging is known as progressive and inevitable process related to the accumulation of oxidative damage into almost all type of biomolecules such as nucleic acids, lipids, proteins or carbohydrates due to an imbalance between pro-oxidants and antioxidants (Mariani et al, 2005). It is also believed that oxidative damage to critical molecules occurs early in the pathogenesis of AD and yield significant neuropathological alterations. Therefore, it represents a potential therapeutic target for slowing the onset and progression of AD (Baldeiras et al, 2008; Lovell and Markesbery, 2007; Nunomura et al, 2001; Solfrizzi et al, 2006). Post-mortem and in vivo studies also show that there are products of free radical damage in the CNS and peripheral tissues of subjects who suffering from AD or MCI (Butterfield et al, 2006; Butterfield et al, 2007; Mangialasche et al, 2009). Regarding peripheral markers of oxidative damage, a few studies have demonstrated that most of the changes found in AD patients are already present in MCI patients (Markesbery and Lovell, 1998; Migliore et al, 2005; Bermejo et al, 2008).
The antioxidant enzymes SOD, catalase, glutathione peroxidase and glutathione reductase, display reduced activities in these diseases (Keller et al, 2005). In addition, markers of lipid peroxidation, such as malondialdehyde (MDA) and intermediate product of nucleic acid modification (8-OHdG) have been found elevated in AD and MCI (Baldeiras et al, 2008; Greilberger et al, 2008; Lovell et al, 1995; Markesbery and Lovell, 1998).

Antioxidant vitamins may reduce neuronal damage and death from oxidative reactions by inhibiting the generation of ROS, lipid peroxidation, apoptosis, protein oxidation, and damage to cell membranes and/or DNA (Maxwell et al, 2005).

### 3.6.2. Oxidative stress in MCI

Free radicals can lead to development of the pathological alterations and cause the neurodegenerative disorder (Baldeiras et al, 2008; Sultana et al, 2008). Generally, ROS is produced by oxidative phosphorylation in which molecular oxygen is converted to water along with the production of ATP. Approximately, 2% of oxygen utilized in the course of oxidative phosphorylation is converted into $O_2^\cdot$ and manganese superoxide dismutase (Mn-SOD) is able to counteract the harmful effects of it and produce H2O2 (Halliwell and Gutteridge, 1999; Preiser JC, 2012). H2O2 can be converted to water by glutathione peroxidase (GSH-Px) or catalase. However, in the presence of redox active metals such as Fe and copper, H2O2 can be converted to hydroxyl via Fenton or Haber-Weiss reactions (Moreira et al, 2005).

Amyloid beta (Ab) as a major component of senile plaques in AD can be an additional source of ROS. A large body of literature shows that Ab mediates oxidative damage to lipids, proteins, and DNA (Moreira et al, 2005).

Increased production of ROS and RNS in MCI might lead to a quick consumption of plasma antioxidants. As mentioned, due to high oxygen consumption rate, limited antioxidant capacity compared with other tissues, high abundance of polyunsaturated fatty acids and the relatively high level of redox active metals, the brain is susceptible to oxidative damage (Berg D, 2007). As depleted, the antioxidant systems are not able to protect the organism against the oxidative damage.
Most studies show increased level of Macromolecules oxidation in MCI and AD diseases:

1. Lipid peroxidation

Lipid peroxidation is one of the major outcomes of free radical-mediated injury to brain (Montine et al, 2004). Several studies have demonstrated that lipid peroxidation is increasing in the brain of MCI and AD subjects compared with healthy controls because brain is rich in polyunsaturated fatty acid. In addition, brain has high concentration of redox transition metal ions and low in antioxidant capacity (Keller et al, 2005; Lovell et al, 1995; Markesbery and Carney, 1999).

Lipid peroxidation is the mechanism by which lipids are attacked by ROS that have sufficient reactivity to abstract a hydrogen atom from a methylene carbon in their side chain. This explains why polyunsaturated fatty acids (PUFA) are particularly susceptible to lipid peroxidation.

Brain membrane phospholipids consist of arachidonic and docosahexaenoic acids. Oxidation of these acids produces aldehydes, among them malondialdehyde and 4-hydroxynonenal are the most investigated.

In autopsied specimens from many regions of brain and in CSF as a source of CNS tissue in subjects with AD, levels of both compounds are increased (Keller et al, 2005; Lovell et al, 1995; Markesbery and Carney, 1999).

Oxidative damage in the brain of subjects suffering from amnestic MCI showed an increase in thiobarbituric acid–reactive substances (21%) and malondialdehyde (60%) in the temporal lobe compared with healthy controls (Keller et al, 2005). Most studies demonstrated increasing of F2-isoprostane levels (a biomarker of lipid peroxidation) in frontal, inferior parietal lobule and occipital regions of patients with MCI compared with age-matched control subjects (Praticò and Sung, 2004).

Lipid peroxidation indicative of oxidative stress can be evaluated by methods including the quantification of peroxidation end products such as MDA (Greilberger et al, 2008).
2. Protein Oxidation

There are few published studies regarding protein oxidation in MCI. ROS cause oxidative damage to proteins. The backbone and their side chain of proteins can be reacted by ROS. The side-chain residues are attacked and many of products are yielded. These products can in turn react with amino acid side chains to produce carbonyl functions (Smith et al, 1991; Hensley et al, 1995).

First study demonstrated an increase in protein carbonyls levels (a marker of protein oxidation) in the superior and middle temporal gyri (A rounded ridge, as on the surfaces of the cerebral hemispheres) in patients with MCI compare with cognitively normal control subjects (Keller et al, 2005). To identify specifically oxidized proteins in AD brain, proteomics can be a developing tool (Butterfield, 2004; Aldred et al, 2004).

Using redox proteomics approach, a study demonstrated oxidation of several specific proteins in the hippocampus (HIP) of a-MCI patients (Sultana et al, 2008). In addition, AD brain has a protein carbonyl end product namely ortho-tyrosine, another marker of protein oxidative damage, similar to controls (Hayn et al, 1996).

A study demonstrates elevation of 3-nitrotyrosine levels (markers of protein oxidation) in brains from subjects with amnestic MCI compared with healthy controls (Butterfield et al, 2007).

3. DNA Oxidation

DNA can be attacked by free radical, especially •OH–, lead to strand breaks, DNA-DNA and DNA protein cross-linking, and modifications of bases. Free radical could contribute to alterations in protein production that further generate neuron dysfunction and death. Generally, several reasons exist that mitochondrial DNA (mtDNA) is more vulnerable to free-radical-mediated damage including: the proximity to ROS production, the lack of protective histones, the limited repair capacity, and the lack of significant regions of noncoding sequences. Oxidized bases represent one of the major classes of DNA lesions induced by ROS (Markesbery and Lovell, 2007).
More than 20 DNA adducts have been recognized, but the most thoroughly studied involves the C8 hydroxylation of guanine and formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG). Former studies have indicated that 8-OHdG is oxidized in both nuclear DNA (nDNA) and mtDNA in late-stage AD (Gabbita et al, 1998; Wang et al, 2005).

8-OHdG level (a marker of DNA oxidation) in nDNA and mtDNA separated from three cortical areas and from cerebellum of MCI and AD patients was increased significantly compared with controls, particularly in the parietal cortex. Levels of 8-hydroxyadenine were significantly increased in frontal, temporal, and parietal lobes in nDNA among MCI patients compared with controls (Wang et al, 2006).

These levels were much higher in mtDNA than in nDNA, showing elevated susceptibility of mitochondria to oxidative stress. In addition, elevated levels of 8-OHdG were also detected in lymphocyte DNA from AD donors (Mecocci et al, 1998). In the only study to quantify oxidized DNA in brain of amnestic MCI patients, they showed that 8-OHdG was significantly elevated in nDNA from frontal and temporal lobes of the patients compared with age-matched controls. The most interesting observation of another study suggested that oxidative modification of nDNA and mtDNA occurs early in the pathogenesis of AD (Wang et al, 2006).
4. Methodology

4.1. Study location:

This study was conducted to investigate the effect of an intervention with antioxidant vitamins E and C on MCI among elderly people in Isfahan, Iran. This city covers an area of approximately 106,179 square kilometer and located about 340 km south of Tehran in center of Iran. The Isfahan metropolitan area had a population of 4,815,863 by 2011 Census, the second most populous metropolitan area in Iran after Tehran. Indeed, in Isfahan, more than 8.12% of the population is currently aged 60 years or older. The population of elderly people in Isfahan is about 391,048.

4.2. Study design

This study was designed as a randomized, double-blind, placebo-controlled trial. Participants were a group of elderly individuals between the ages of 60-75 years. They were recruited from the retirees clubs. There are seven retirees clubs in Isfahan city and more than 45000 members have registered in these clubs. At the beginning of the trial, through advertising, the volunteers who would like to participate in the study were registered. After completion the list of the cases from retirees clubs, they were interviewed by telephone to find those who met the criteria. Then, an expert psychologist evaluated the subjects to find those who suffering from Mild Cognitive Impairment. The MMSE was used to assess cognitive functions, and the score of 21-26 was indicative of mild cognitive impairment (Lopez et al, 2005). MCI patients referred to a local clinical laboratory for routine biochemical and hematological evaluation.

The Iranian version of MMSE was validated and found to be suitable for usage among the local elderly population (Seyedian et al, 2008). The characteristic of the sample are described in Sampling Criteria.

Two hundred and fifty six elderly volunteers who met the criteria (explained below) were randomly assigned into two groups (supplemented and control). There were 127 in supplemented and 129 in control group. The first group consumed 300 mg of
vitamin E (DL-α-tocopheryle acetate) plus 400 mg vitamin C (Ascorbic acid) per day, the second group consumed placebo with the identical condition and they completed the 1-year intervention period. Vitamin E was given as a single dose in the morning together with a meal. Vitamin C was given as two doses of 250 mg in the morning and evening throughout the week except Fridays. The study was double blind for patients and researcher to reduce the possibility of bias. Selection and grouping was performed using a stratified randomization. The MCI subjects were divided to two groups according to their gender (male or female), each of them were further divided to three age groups including 60-65, 65-70, and 70-75 years. The subjects within each of these six groups were then further divided to two equally numbered supplemented or placebo subgroups by simple randomization (figure 5).

*Figure 5. Stratified and simple randomization (1)*
4.3. Sample size

This study is including 3 age groups 60-64, 65-69, and 70-75. Primary studies have shown that the standard deviation of MMSE score among elderly people who have MCI for each age group is about 2 (Seyediam et al, 2008). Studies have also shown that mean score of MMSE in these people is about 23.5 (Lopez et al, 2005). We expect after consuming of the vitamin E and C, their MMSE score will be 25 or more (Ibáñez-Hernández et al, 2008). Therefore, based on the equation below, the sample size needed for this study was estimated to be at least 37 in each age group, 111 for each of supplemented and control group and a total of 222 samples for all. Assuming \( \alpha = 0.05, \beta = 0.1, 1 - \beta = 0.90 \), the sample size was determined. The sample size was calculated based on the equation below:

\[
N = \frac{2(Z_{1-\alpha/2} + Z_{1-\beta})^2 \times \sigma^2}{(\mu_0 - \mu_1)^2 (1.96+1.28)^2 \times 2^2} = 37
\]

Considering probable withdraw rate of the subjects during the study, we multiplied the sample size by 1.5.

4.4. Sampling Criteria

The inclusion and exclusion criteria of this study were as follow:

**Inclusion criteria:**
- Age between 60-75 years old
- A minimum educational level of fifth grade.
- MMSE score between 21-26
Exclusion criteria:
- Having Dementia, Depression or Epilepsy
- Having mental retardation, history of brain surgery or any significant neurological disease
- Having severe Cardiovascular Disease (CVD) and severe anemia
- Having severe kidney or liver disease, inflammatory intestinal disease and any of diseases that interfere with the antioxidant’s absorption
- Consumption of antioxidant medication or vitamin that might modify the results
- Body mass index (BMI) more than 30 kg/m²
- Having special diet (vegetarian, vegan, etc.)
- Smoking
- Addicted to alcohol
- Using neuroleptic drugs, benzodiazepine, immunosuppressant, anti-depression and anticonvulsants medication
- Current medication known to influence vitamin E and C status (laxatives and hormone replacement therapy)

The study was conducted according to provisions of the Helsinki declaration and the local ethics committee at Ministry of Health and Medical Education (Iran) approved the study. All the subjects or their families signed the consent for the participation in this study.

4.5. Data collection

The subjects were undergone laboratory examination at three time points during this study, i.e., at the beginning of the study, six month after intervention, and at the end of intervention. Information on background characteristics, anthropometric measurements and blood pressure at baseline and food consumption (through Three-Day Diet Recall) one time every two months were gathered. The subjects were invited by telephone to receive their free tablets (vitamin E and C) monthly during the
intervention. The results of their blood test were given three times as free during the project.

4.6. Background characteristics
Information on background characteristics including health history, family history of dementia, use of medications, tobacco and other lifestyle factors were collected. Medicine consumption is common in elderly individuals thus; current use of their medicine was recorded at the same time as assessment of dietary record. Effects of Medicines were eliminated by proper statistical tests.

4.7. Anthropometric and blood pressure measurements
Anthropometric and blood pressure measurements of the subjects were taken at baseline. Body weight and height were measured with Seca scale and non-stretchable tape. Body weights with 0.1 kg accuracy without shoes and with minimum clothing were measured.
Heights with 0.1 cm accuracy were also measured. BMI was determined by dividing body weight by height squared (kg/m$^2$). Blood pressure (BP) is determined by using a sphygmomanometer (an inflatable cuff equipped with a manometer to display the pressure), and then monitoring the flow of blood through the artery with a stethoscope as the cuff pressure is allowed to slowly decline. The pressure at which the first thumping sounds of blood flowing under the cuff marks systole, and the point at which these sounds become muffled is diastole.

4.8. Dietary records
A three day food record to get an accurate description of their typical daily diet for three consecutive days was designed. The day included two weekdays and one weekend day. At baseline, the participants were taught by a dietician how to record the amounts of foods that were eaten. A written instruction including of amount and conversion was also given to them.
Every two months, when the three day food record form was completed, subjects returned it personally. Nearly, every subject completed 18 food records throughout the study. A trained and qualified nutritionist inspected all records to ensure that they were completed and that adequate detail had been recorded. The amount of food eaten by each subject was estimated from household measures. The nutrient contents (vitamin C, E and others) of all foods ingested using the computer based program, Food Processor II (ESHA, Salem, OR, USA) were determined. The database of this software is built upon the Nutrient Database Bank for Standard Reference from the US Department of Agriculture and other sources. The database was modified with reference to the existing national Iranian food composition table, developed by the Iranian National Institute of Nutrition and Food Technology.

4.9. Cognition Function

Evaluation of cognitive function by Mini-Mental State Examination (MMSE) was blindly performed by an expert psychologist. MMSE is a widely recognized tool for evaluation of cognitive impairment with numerous translations and adaptations. This test comprises 11 questions concerning orientation in place and time, immediate and delayed recall of three words, attention and calculation, language and praxis, and visual construction. This questionnaire has been translated and validated for the Iranian population (Seiedian et al, 2007). Scores can range from zero to 30 and the score of 21-26 was indicative of mild cognitive impairment. A total score is calculated by summing the number of words mentioned for each letter.

4.10. Biochemical Measurements

All cases underwent a thorough biochemical evaluation. 10-11 ml blood samples were taken from the antecubital vein in the morning from subjects who had fasted overnight. Blood sample were drawn at three stages on days 0, 180 and 360 after intervention. Samples were placed in vacutainer tubes without anticoagulant allowed
to clot and centrifuged immediately. Sera were aliquoted into 0.5 ml Eppendorf tubes and stored at -80°C until measurement to avoid repeated serum defrosting. For determination of total Glutathione (GSH) whole blood was used.

The serum samples were analyzed for:

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>HPLC</td>
</tr>
<tr>
<td>TAC</td>
<td>Spectrophotometric</td>
</tr>
<tr>
<td>GSH</td>
<td>Spectrophotometric</td>
</tr>
<tr>
<td>8-OHDG</td>
<td>Elisa</td>
</tr>
</tbody>
</table>

### 4.10.1. Malondialdehyde (MDA)

Serum MDA was measured according to the method described by Yu et al, (Yu et al, 1986). Standards and samples were placed in a boiling water bath at 100°C in the presence of thiobarbituric acid (TBA) for 1 hour. The reaction was stopped with ice water. Levels of lipid peroxidation were measured by the formation of a thiobarbituric acid (TBA) adduct of malondialdehyde (MDA), separated by HPLC. Using an analytical column Spherisorb ODS2 5 μm (250 × 4.6 mm), eluted with 60% (v/v) potassium phosphate buffer 50 mM, pH 6.8, and 40% (v/v) methanol at a flow rate of 1mL/min. Spectrophotometric detection of the TBA- MDA adducts occurred at excitation/emission 563/532 nm.

### 4.10.2. Glutathione (GSH)

GSH was measured quantitatively at a Perkin-Elmer spectrophotometer according to the method of Beutler et al (Beutler et al, 1963). Briefly, glutathione were extracted after hemolysis of RBCs followed by precipitation of hemolysate by Metaphosphoric Na2EDTA saline solution. 0.2 ml of whole blood were added to 1.8 ml distilled water and 3 ml precipitating solution and let to remain at room temperature for 5 minutes. Precipitating solution include Metaphosphoric acid, Na2EDTA and NaCl. Hemoglobin solutions were then filtrated through Whatman paper grade No.1.
2 ml of filtrate were added to 8 ml phosphate di-sodium hydrogen phosphate (42.6 g/l). Yellow color developed by adding 5, 5’-dithiobis (2-nitrobenzoic acid) (DTNB) to Sulfhydryl Compounds. DTNB known as Ellman’s Reagent, was developed for the detection of thiol compounds.

The intensity of developed color was determined by reading at 412 nm against reagent blank. GSH concentrations by using optical density/concentration standard curve were calculated. The results were expressed as μmol GSH/gHb.

Hemoglobin concentrations of whole blood by K-1000 sysmex hematologic autoanalyzer were measured.

4.10.3. Total Antioxidant Capacity (TAC)

Total antioxidant capacity (TAC) was measured according to Miller and Rice-Evans (Miller and Rice-Evans, 1993). The assay is based on the hindrance of the absorbance of radical cations of 2, 2’-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) by antioxidants, produced as a result of incubation of ABTS with a peroxidase such as Metmyoglobin and H2O2.

ABTS solution was prepared by dissolving 27.43 mg ABTS in 10 ml of PBS buffer and was protected from light (PH= 7.4). A fresh ABTS stock solution was prepared every day. PBS containing 8.2 g NaCl, 0.2 g KCl, 1.2 g di-sodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate fills up with distilled water to 1L and was stored in room temperature.

Fresh working standards (0.5, 1, 1.5, 2 mM) are prepared daily by mixing 2.5 mM Trolox (a water-soluble derivative of vitamin E) with PBS.

Before measuring, buffer and ABTS are tempered at 30°C.

The reagents are mixed as follows: 400 μl buffer (410 μl for measuring the blank), 10 μl standard (20 μl sample), 20 μl metmyoglobin and 400 μl ABTS, with vortexing.

The reaction is started by the addition of 170 μl of 450 μM H2O2, the clock started, and the tube mixed. A quantitative relationship exists between the absorbance at 734 nm at 6 min and the antioxidant activity of the sample or standard.
4.10.4. 8-Hydroxydeoxyguanosine (8-OHDG)

8-OHdG was measured by using a competitive in vitro enzyme-linked immunosorbent assay (ELISA) kit from Immundiagnostik Company, Germany. Briefly, the interfering substances were first eliminated by ultrafiltration filter (cut off molecular weight 10,000). Pre-treat ultra-filter followed to the maker’s manuals. 8-OHdG monoclonal antibody and serum samples are added to microtiter plate per coated with 8-OHdG. The serum 8-OHdG content compete with coated 8-OHdG for binding to monoclonal antibody. The more 8-OHdG present in serum, the less monoclonal antibody binds to wells. After washing and addition of an enzyme-labeled secondary antibody and then chromatic substrate the resulting, color density of the plate were measured. The color density is reversed to samples 8-OHdG concentration.

4.11. Statistical analysis

The results are presented as mean ± standard error (SE). Repeated measures analysis of variance (ANOVA) was the main statistical method used for analyzing data. This test permits examination at each time point. Mauchly’s sphericity test was conducted to assess sphericity as a perquisite assumption. When the assumption was not satisfied, Huynh-Feldt correction was used. Within group comparisons at each follow-up time point were made using repeated contrasts. Between groups comparisons were conducted using independent samples t-test. Chi square test for difference between proportions was used. Analysis of the data was done by SPSS software version 19. Analysis of consumed foods was done by Food Processor II software. For all hypotheses, a significance level of P<0.05 was considered statistically significant.
5 Results

Based on our study design, data were presented in four sections:

5-1 General characteristic of the subjects
5-2 Oxidative stress biomarkers
5-3 Mini-Mental State Examination (MMSE) scores
5-4 Dietary intakes of the subjects

At baseline, the total number of subjects who registered in this study was 761. All of them were assessed by MMSE questionnaire in order to find the participants with MCI. Of the 761 volunteers, 296 were found to have MCI and selected for next part of this study.

Of these, 40 did not continue with the study, due to one of the following reasons:

1 subject was died.
6 subjects went to a long trip.
14 subjects didn’t tolerate consuming the supplementation.
19 subjects refused to participate.

Therefore, 256 subjects remained till the end of the study (figure 6).
Figure 6. Stratified and simple randomization (2)
5.1. Characteristics of participants in baseline

Socio-demographic characteristics of the studied subjects were compared in supplemented and control groups in order to find whether there are any significant differences among these characteristics. As table 5.1.1 shows no significant differences were found between supplemented and control group in terms of male and female at baseline (P< 0.229).

Table 5.1.1. The number and percentage of males and females in the two groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Male</td>
<td>63</td>
<td>49.6</td>
</tr>
<tr>
<td>Female</td>
<td>64</td>
<td>50.4</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>100</td>
</tr>
</tbody>
</table>

Participants were a group of elderly individuals between the ages of 60 and 75 years. Table 5.1.2 shows the distribution of the participants by age groups. No significant differences were found between supplemented and control groups for the age distributions (P<0.975).

Table 5.1.2. Distribution of the studied subjects for three age subpopulations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>supplemented</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Age group</td>
<td>60-64</td>
<td>48</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>45</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>34</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>127</td>
<td>100</td>
</tr>
</tbody>
</table>

The means and standard error (SE) of age, weight, height, BMI, systolic and diastolic blood pressure of the subjects at the baseline are presented in Table 5.1.3. Body Mass
Index (BMI) is an anthropometric index of weight and height (stature) that is defined as body weight in kilograms divided by height in meters squared.

The desirable range for BMI is between 20 and 25. The values above 25 and 35 are overweight and obese, respectively. In this study, the mean values of BMIs were within the overweight range for two groups at baseline.

As the results in table 5.1.3 shows, there was no statistically significant difference among these variables between supplemented and control group at the beginning of the study.

### Table 5.1.3. Means and standard error (SE) of characteristics of the subjects at baseline

<table>
<thead>
<tr>
<th>Variables</th>
<th>Supplemented (n=127)</th>
<th>Control (n=129)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>66.5±0.39</td>
<td>66.3±0.38</td>
<td>0.755</td>
</tr>
<tr>
<td>Weight</td>
<td>71.0±0.94</td>
<td>69.1±0.94</td>
<td>0.139</td>
</tr>
<tr>
<td>Height</td>
<td>162.5±0.78</td>
<td>162±0.86</td>
<td>0.703</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.9±0.35</td>
<td>26.3±0.35</td>
<td>0.233</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>141.2±1.67</td>
<td>137.4±1.7</td>
<td>0.119</td>
</tr>
<tr>
<td>Diastolic Blood Pressure</td>
<td>80.2±1.2</td>
<td>77.9±1.2</td>
<td>0.164</td>
</tr>
</tbody>
</table>

Means and standard error of characteristics for three age groups has been shown in table 5.1.4. The ANOVA did not show any difference among the three age subpopulation at baseline for weight, height, BMI, systolic and diastolic blood pressure.
Table 5.1.4. Means and standard error (SE) of characteristics for three age subpopulations at baseline

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>Supplemented (n=127)</th>
<th>Control (n=129)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>60-64</td>
<td>72.3±1.7</td>
<td>69.3±1.6</td>
<td>0.215</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>71.7±1.6</td>
<td>68.8±1.6</td>
<td>0.210</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>68.4±1.3</td>
<td>68.9±1.6</td>
<td>0.780</td>
</tr>
<tr>
<td>Height</td>
<td>60-64</td>
<td>161.7±1.4</td>
<td>162±1.3</td>
<td>0.871</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>162.9±1.2</td>
<td>161.6±1.6</td>
<td>0.517</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>162.9±1.5</td>
<td>162.7±1.7</td>
<td>0.890</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>60-64</td>
<td>27.8±0.69</td>
<td>26.4±0.54</td>
<td>0.122</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>27.0±0.50</td>
<td>26.4±0.59</td>
<td>0.465</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>25.8±0.52</td>
<td>26.2±0.72</td>
<td>0.633</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>60-64</td>
<td>136.6±2.8</td>
<td>132.0±2.6</td>
<td>0.213</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>141.8±2.70</td>
<td>142.3±2.8</td>
<td>0.898</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>146.7±3.2</td>
<td>139.0±3.5</td>
<td>0.104</td>
</tr>
<tr>
<td>Diastolic Blood Pressure</td>
<td>60-64</td>
<td>79.0±14.3</td>
<td>78.1±1.6</td>
<td>0.708</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>80.5±1.75</td>
<td>77.4±2.3</td>
<td>0.296</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>81.4±2.2</td>
<td>78.1±2.4</td>
<td>0.325</td>
</tr>
</tbody>
</table>

Table 5.1.5 and 5.1.6 show the level of literacy for all and three age subpopulations of the subjects respectively. Most subjects in the present study were completed high school diploma and university degree while the least were at secondary level. There was no significant difference in the level of literacy among all study groups at baseline (P<0.127) except a significant difference in the level of secondary in terms of age groups of 70-75 years (P<0.026).

Table 5.1.5. Level of literacy among subjects in studied subjects at baseline

<table>
<thead>
<tr>
<th>Education</th>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Primary</td>
<td>25</td>
<td>19.8</td>
<td>16</td>
</tr>
<tr>
<td>Secondary</td>
<td>17</td>
<td>13.5</td>
<td>10</td>
</tr>
<tr>
<td>Diploma</td>
<td>48</td>
<td>38.1</td>
<td>55</td>
</tr>
<tr>
<td>University degree</td>
<td>36</td>
<td>28.6</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>100</td>
<td>128</td>
</tr>
</tbody>
</table>
Table 5.1.6. Level of literacy for three age subpopulations in studied subjects at baseline

<table>
<thead>
<tr>
<th>Education</th>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Primary</td>
<td>60-64</td>
<td>11</td>
<td>73.3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>6</td>
<td>60.0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>8</td>
<td>50.0</td>
<td>8</td>
</tr>
<tr>
<td>Secondary</td>
<td>60-64</td>
<td>3</td>
<td>37.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>6</td>
<td>54.5</td>
<td>5</td>
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<tr>
<td></td>
<td>70-75*</td>
<td>8</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Diploma</td>
<td>60-64</td>
<td>23</td>
<td>54.8</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>18</td>
<td>43.9</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>7</td>
<td>35.0</td>
<td>13</td>
</tr>
<tr>
<td>University degree</td>
<td>60-64</td>
<td>11</td>
<td>34.4</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>14</td>
<td>50.0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>11</td>
<td>47.8</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>60-64</td>
<td>48</td>
<td>100</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>44</td>
<td>100</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>34</td>
<td>100</td>
<td>33</td>
</tr>
</tbody>
</table>

*Chi square test

As table 5.1.7 shows, the percentage of dementia among close relative of participants (a close relative is defined as a parents, brother or sister) in supplemented and control group was almost similar (13.7 VS. 15.2). There was no statistical significant difference in dementia among close relative of study subjects between two groups at baseline (P<0.663).

Table 5.1.7. Distribution of the studied subjects by occurrence of dementia among close relative at baseline

<table>
<thead>
<tr>
<th>Dementia among close relative</th>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>No</td>
<td>107</td>
<td>86.3</td>
<td>106</td>
<td>84.8</td>
</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>13.7</td>
<td>19</td>
<td>15.2</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>100</td>
<td>125</td>
<td>100</td>
</tr>
</tbody>
</table>
Occurrence of dementia among close relative of study subjects for three age subgroups are presented in table 5.1.8. The Chi-Square test did not show any difference among the three age subgroups at baseline.

Table 5.1.8. Distribution of the studied subjects by occurrence of dementia among close relative for three age subpopulations at baseline

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dementia</th>
<th>supplemented</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>No</td>
<td>60-64</td>
<td>41</td>
<td>50.6</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>35</td>
<td>46.1</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>31</td>
<td>55.4</td>
<td>25</td>
</tr>
<tr>
<td>Yes</td>
<td>60-64</td>
<td>6</td>
<td>40.0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>9</td>
<td>69.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>2</td>
<td>25.0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>60-64</td>
<td>47</td>
<td>100</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>44</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>33</td>
<td>100</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 5.1.9 shows some other variables including marital status, household size and aerobic exercise which can also act as confounding factors. 82.5% and 74.2% of elderly individuals in the supplemented and control were married respectively. The percentage of single and divorce were 0.8% and 2.4% in supplemented group respectively. Size of household with more than five people was 1.6 and 2.3 % in supplemented and control respectively. More than 62 % of individuals in each group refused from aerobic exercise.

Tables 5.1.10, 5.1.11 and 5.1.12 show some of the variables including marital status, household size and aerobic exercise for three age subgroups separately.

None of the variables showed any significant differences between two study groups, and also for any of their three age subgroups.
### Table 5.1.9. Distribution of the studied subjects for some other variables at baseline

<table>
<thead>
<tr>
<th>Other variables</th>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Marital Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Married</td>
<td>104</td>
<td>82.5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>1</td>
<td>0.8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Death of husband or wife</td>
<td>18</td>
<td>14.3</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Divorce</td>
<td>3</td>
<td>2.4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>126</td>
<td>100</td>
<td>128</td>
</tr>
<tr>
<td>Household size</td>
<td>&lt;=5</td>
<td>124</td>
<td>98.4</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>&gt;5</td>
<td>2</td>
<td>1.6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>136</td>
<td>100</td>
<td>128</td>
</tr>
<tr>
<td>Aerobic exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>88</td>
<td>69.8</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Occasionally</td>
<td>23</td>
<td>18.3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Systematically</td>
<td>15</td>
<td>12.0</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>126</td>
<td>100</td>
<td>128</td>
</tr>
</tbody>
</table>

### Table 5.1.10. Distribution of the studied subjects by marital Status for three age subpopulations at baseline

<table>
<thead>
<tr>
<th>Marital status</th>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Married</td>
<td>60-64</td>
<td>40</td>
<td>83.3</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>36</td>
<td>81.8</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>28</td>
<td>82.4</td>
<td>23</td>
</tr>
<tr>
<td>Single</td>
<td>60-64</td>
<td>1</td>
<td>2.1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Death of husband or wife</td>
<td>60-64</td>
<td>5</td>
<td>10.4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>7</td>
<td>15.9</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>6</td>
<td>17.6</td>
<td>7</td>
</tr>
<tr>
<td>Divorce</td>
<td>60-64</td>
<td>2</td>
<td>4.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>1</td>
<td>2.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>60-64</td>
<td>48</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>44</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>34</td>
<td>100</td>
<td>33</td>
</tr>
</tbody>
</table>
Table 5.1.11. Distribution of the studied subjects by household size for three age subpopulations at baseline

<table>
<thead>
<tr>
<th>Household size</th>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>&lt;=5</td>
<td>60-64</td>
<td>47</td>
<td>97.9</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>44</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>33</td>
<td>97.1</td>
<td>33</td>
</tr>
<tr>
<td>&gt;5</td>
<td>60-64</td>
<td>1</td>
<td>2.1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>1</td>
<td>2.9</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>60-64</td>
<td>48</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>44</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>34</td>
<td>100</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 5.1.12. Distribution of the studied subjects by aerobic exercise for three age subpopulations at baseline

<table>
<thead>
<tr>
<th>Aerobic Exercise</th>
<th>Groups</th>
<th>supplemented</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>No</td>
<td>60-64</td>
<td>32</td>
<td>66.7</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>31</td>
<td>70.5</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>25</td>
<td>73.5</td>
<td>23</td>
</tr>
<tr>
<td>Occasionally</td>
<td>60-64</td>
<td>8</td>
<td>16.7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>9</td>
<td>20.5</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>6</td>
<td>17.6</td>
<td>5</td>
</tr>
<tr>
<td>Systematically</td>
<td>60-64</td>
<td>8</td>
<td>16.7</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>4</td>
<td>9.1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>3</td>
<td>8.8</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>60-64</td>
<td>48</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>44</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>34</td>
<td>100</td>
<td>33</td>
</tr>
</tbody>
</table>
5.2. Oxidative stress biomarkers

5.2.1. Malondialdehyde (MDA)

To identify any difference within and between supplemented and control groups for MDA level among all subjects and different age groups at three time points, repeated measure ANOVA was applied. Results of within and between groups for the Malondialdehyde (MDA) among all subjects are illustrated in table 5.2.1.1. The repeated measure ANOVA indicated that the difference within supplemented and control groups was significant ($P<0.001$). Comparison between two groups showed significant differences ($P<0.002$). Multivariate test of time $\times$ treatment interaction (Wilks' $\lambda$) indicated that this interaction for MDA was not statistically significant. Mean values of MDA in supplemented group in six$^{th}$ month ($2.37\pm0.055$, $P<0.02$) and twelv$^{th}$ month ($1.75\pm0.067$, $P<0.001$) were significantly different from control group ($2.54\pm0.055$ and $2.17\pm0.089$) respectively.

Table 5.2.1.1. Results of within and between groups comparisons for the Malondialdehyde (MDA) based on repeated measure ANOVA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time $\times$ treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde (MDA) (nmol/ml)</td>
<td>Supplemented</td>
<td>1.54±0.054</td>
<td>2.37±0.055</td>
<td>1.75±0.067</td>
<td>F=153.7</td>
<td>F=9.49</td>
<td>F=2.44</td>
</tr>
<tr>
<td>Control</td>
<td>1.56±0.052</td>
<td>2.54±0.055</td>
<td>2.17±0.089</td>
<td>F=9.49</td>
<td>P&lt;0.001**</td>
<td>P&lt;0.002*</td>
<td>P&lt;0.12</td>
</tr>
<tr>
<td>$t$ value</td>
<td>$t=0.27$</td>
<td>$t=2.3$</td>
<td>$t=3.8$</td>
<td>$t=2.3$</td>
<td>$t=3.8$</td>
<td>$t=3.8$</td>
<td>$t=3.8$</td>
</tr>
<tr>
<td>$p$ value</td>
<td>$P&lt;0.78$</td>
<td>$P&lt;0.02*$</td>
<td>$P&lt;0.001*$</td>
<td>$P&lt;0.78$</td>
<td>$P&lt;0.02*$</td>
<td>$P&lt;0.001*$</td>
<td>$P&lt;0.12$</td>
</tr>
</tbody>
</table>

* One way ANOVA  ** Repeated measure ANOVA
Figure 7 shows an increasing trend in MDA level in both groups during the first six months, which followed by a decline in second half year of the study. This decline was significantly different in supplemented compared to the control group at second and third time points.

**Figure 7.** Mean and SEM of MDA (nmol/ml) in the two studied groups at three time points

*P<0.02, significantly different from supplemented group at sixth month point
**P<0.001 significantly different from supplemented group at 12th month point

Results of within and between groups comparisons and also time × treatment interaction for the MDA among three age groups are presented in table 5.2.1.2. In this table, the repeated measure ANOVA showed differences within group in all age subgroups (P<0.001) and between supplemented and control groups in age subgroup of 60-64 and 70-75 years (P< 0.035, P< 0.032, respectively) were significant. In age subgroup of 65-69 year, there was no significant difference between supplemented and control groups. Time × treatment interaction for MDA was not statistically significant among three age groups.
Table 5.2.1.2. Results of within and between groups comparisons for the Malondialdehyde (MDA) among three age groups based on repeated measure ANOVA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/ml) (60-64 years)</td>
<td>Supplemented</td>
<td>1.50±0.083</td>
<td>2.33±0.082</td>
<td>1.74±0.105</td>
<td>F=70.21</td>
<td>P&lt;0.001*</td>
<td>F=1.98</td>
</tr>
<tr>
<td>Control</td>
<td>1.51±0.073</td>
<td>2.53±0.095</td>
<td>2.13±0.135</td>
<td>P&lt;0.035**</td>
<td>P&lt;0.023**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td>t=0.040</td>
<td>t=0.911</td>
<td>t=2.352</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>P&lt;0.968</td>
<td>P&lt;0.365</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (nmol/ml) (65-69 years)</td>
<td>Supplemented</td>
<td>1.54±0.09</td>
<td>2.39±0.084</td>
<td>1.83±0.123</td>
<td>F=53.8</td>
<td>F=2.21</td>
<td>F=0.753</td>
</tr>
<tr>
<td>Control</td>
<td>1.59±0.09</td>
<td>2.52±0.084</td>
<td>2.17±0.167</td>
<td>P&lt;0.01**</td>
<td>P&lt;0.452</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td>t=0.333</td>
<td>t=1.126</td>
<td>t=1.653</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>P&lt;0.740</td>
<td>P&lt;0.264</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (nmol/ml) (70-75 years)</td>
<td>Supplemented</td>
<td>1.56±0.100</td>
<td>2.37±0.130</td>
<td>1.63±0.113</td>
<td>F=28.5</td>
<td>F=4.83</td>
<td>F=2.33</td>
</tr>
<tr>
<td>Control</td>
<td>1.56±0.112</td>
<td>2.56±0.106</td>
<td>2.20±0.162</td>
<td>P&lt;0.01**</td>
<td>P&lt;0.032**</td>
<td>P&lt;0.107**</td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td>t=0.031</td>
<td>t=1.105</td>
<td>t=2.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>P&lt;0.976</td>
<td>P&lt;0.274</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One way ANOVA   ** Repeated measure ANOVA

Figures 8, 9 and 10 also indicate a similar pattern of fluctuation in MDA levels for three age groups.
Figure 8. Mean and SEM of MDA (nmol/ml) in the two studied groups among subjects aged 60-64 years at three time points

Figure 9. Mean and SEM of MDA (nmol/ml) in the two studied groups among subjects aged 65-69 years at three time points

Figure 10. Mean and SEM of MDA (nmol/ml) in the two studied groups among subjects aged 70-75 years at three time points
5.2.2 Total Antioxidant Capacity (TAC)

As shown in table 5.2.2.1, there was an increase in Total Antioxidant Capacity in two groups. By conducting the repeated measure ANOVA, the differences within both group was significant (P< 0.001). Within control group for successive time points using repeated contrasts, showed significant difference only between baseline and six\textsuperscript{th} month (P<0.001), while in supplemented group, significant differences were observed at all-time points(P<0.001). This test also showed statistically significant differences between two groups (supplemented and control) (F = 16.77; P<0.001). There was also a statistically significant interaction between time and treatment (P< 0.001).

As shown in the table 5.2.2.1, the highest increase in mean values was in the supplemented group in twelfth month (1.78±0.048, P<0.001) compared with the control group (1.49±0.031) and there was significant difference between two groups in the end of intervention.

Table 5.2.2.1. Results of within and between groups comparisons for the Total Antioxidant Capacity (TAC) based on repeated measure ANOVA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time \times treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Antioxidant Capacity (TAC) (mM trolox eq)</td>
<td>Supplemented</td>
<td>1.08±0.022</td>
<td>1.47±0.028</td>
<td>1.78±0.048</td>
<td>F=241.0</td>
<td>P&lt;0.001**</td>
<td>F=16.77 P&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.06±0.019</td>
<td>1.43±0.027</td>
<td>1.49±0.031</td>
<td>F=16.38</td>
<td>P&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>\textit{t}-value \textit{p}-value</td>
<td>t=652</td>
<td>t=1.02</td>
<td>t=5.17</td>
<td>P&lt;0.51</td>
<td>P&lt;0.31</td>
<td>P&lt;0.001*</td>
<td></td>
</tr>
</tbody>
</table>

* One way ANOVA    ** Repeated measure ANOVA
Figure 11 shows an ascending trend of TAC in both groups but in supplemented group compared with the control was significant only in the third time point of the study.

**Figure 11.** Mean and SEM of TAC (mM trolox eq) in the two studied groups at three time points

**P<0.001** significantly different from supplemented group at 12th month point

As shown in table 5.2.2.2 which is classified by three age subgroups, similar to table 5.2.2.1, the test shows significant differences within (P<0.001) and between groups in terms of all age groups. Also, the interaction between time and treatment was different significantly. Mean values of TAC also show that there was a significant difference between supplemented and control at 12th month among all three age subgroups.
Table 5.2.2.2. Results of within and between groups comparisons for the Total Antioxidant Capacity (TAC) among three age groups based on repeated measure ANOVA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAC (mM trolox eq) (60-64 years)</td>
<td>Supplemented</td>
<td>1.05±0.036</td>
<td>1.47±0.053</td>
<td>1.77±0.071</td>
<td>F=113.3</td>
<td>F=4.94</td>
<td>F=8.74</td>
</tr>
<tr>
<td>Control</td>
<td>1.08±0.024</td>
<td>1.42±0.045</td>
<td>1.46±0.045</td>
<td>P&lt;0.001**</td>
<td>P&lt;0.029 **</td>
<td>P&lt;0.001 **</td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td>t=0.634</td>
<td>t=-0.613</td>
<td>t=-3.59</td>
<td>P&lt;0.528</td>
<td>P&lt;0.541</td>
<td>P&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.528</td>
<td>&lt;0.541</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAC (mM trolox eq) (65-69 years)</td>
<td>Supplemented</td>
<td>1.10±0.044</td>
<td>1.47±0.040</td>
<td>1.76±0.090</td>
<td>F=67.1</td>
<td>F=5.15</td>
<td>F=5.43</td>
</tr>
<tr>
<td>Control</td>
<td>1.06±0.037</td>
<td>1.43±0.047</td>
<td>1.54±0.054</td>
<td>P&lt;0.001**</td>
<td>P&lt;0.026 **</td>
<td>P&lt;0.007 **</td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td>t=-.0665</td>
<td>t=-0.754</td>
<td>t=-2.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.508</td>
<td>&lt;0.453</td>
<td>&lt;0.042*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAC (mM trolox eq) (70-75 years)</td>
<td>Supplemented</td>
<td>1.09±0.032</td>
<td>1.46±0.048</td>
<td>1.80±0.089</td>
<td>F=60.6</td>
<td>F=7.08</td>
<td>F=8.34</td>
</tr>
<tr>
<td>Control</td>
<td>1.03±0.038</td>
<td>1.43±0.046</td>
<td>1.43±0.062</td>
<td>P&lt;0.001**</td>
<td>P&lt;0.01 **</td>
<td>P&lt;0.001 **</td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td>t=-1.249</td>
<td>t=-0.351</td>
<td>t=-3.37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.216</td>
<td>&lt;0.727</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One way ANOVA ** Repeated measure ANOVA

Figure 12, 13 and 14 show the TAC levels in three age groups over the three time points. As shown in the figures, the mean values of TAC were significantly different between supplemented and control group only in twelfth month.
Figure 12. Mean and SEM of TAC (mM trolox eq) in the two studied groups among subjects aged 60-64 years at three time points.

Figure 13. Mean and SEM of TAC (mM trolox eq) in the two studied groups among subjects aged 65-69 years at three time points.

Figure 14. Mean and SEM of TAC (mM trolox eq) in the two studied groups among subjects aged 70-75 years at three time points.
5.2.3 Glutathione (GSH)

The result of within and between groups for the Glutathione (GSH) using repeated measure ANOVA is presented in table 5.2.3.1. Within supplemented group for successive time points using repeated contrasts, showed significant differences between baseline and six\textsuperscript{th} month (P<0.02), while in control group, no significant differences were observed at all-time points. The interaction between time and treatment was significant between sixth and twelfth month of intervention (P<0.02).

Mean values of the GSH showed no significant difference between two groups at baseline and 6\textsuperscript{th} month of intervention (table 5.2.3.1) but there was a significant difference between supplemented and control group at twelf\textsuperscript{th} month of intervention (64.5±1.38, 59.7±1.2, P<0.01).

Table 5.2.3.1. Results of within and between groups comparisons for the Glutathione (GSH) based on repeated measure ANOVA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione (GSH) (nM/g Hb)</td>
<td>Supplemented</td>
<td>61.7±2.84</td>
<td>58.9±1.25</td>
<td>64.5±1.38</td>
<td>F=1.683</td>
<td>F=0.236</td>
<td>F=5.580 **</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>59.7±2.27</td>
<td>60.9±1.31</td>
<td>59.7±1.25</td>
<td>t=-0.529</td>
<td>P&lt;0.59</td>
<td>P&lt;0.63</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=1.23</td>
<td>t=-2.52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.22</td>
<td>P&lt;0.01 *</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
* One way ANOVA
** Repeated measure ANOVA
Figure 15 shows a fluctuation in the trend of GSH in two groups but only in supplemented group compared the control was significant in the third time point of the study.

**Figure 15.** Mean and SEM of GSH (nM/g Hb) in the two studied groups at three time points

**P<0.01 significantly different from supplemented group at 12th month point**

Repeated measures ANOVA revealed no significant increasing in GSH between supplemented and control among three age subgroups over the follow-up period (table 5.2.3.2). By conducting the one way ANOVA, mean differences of GSH in the second time point of the study among 65-69 years (P<0.053) and third time point among 70-75 years (P<0.032) were significant.
Table 5.2.3.2. Results of within and between groups comparisons for the Glutathione (GSH) among three age groups based on repeated measure ANOVA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>Supplemented</td>
<td>60.02±4.39</td>
<td>56.64±2.04</td>
<td>62.35±2.52</td>
<td>F=0.410</td>
<td>F=0.712</td>
<td>F=0.445</td>
</tr>
<tr>
<td>(nM/g Hb)</td>
<td>Control</td>
<td>59.67±3.87</td>
<td>60.03±2.36</td>
<td>60.03±1.87</td>
<td>P&lt;0.665</td>
<td>P&lt;0.401</td>
<td>P&lt;0.591</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=-0.061</td>
<td>t=1.08</td>
<td>t=-0.745</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.951</td>
<td>P&lt;0.284</td>
<td>P&lt;0.458</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>Supplemented</td>
<td>61.27±5.13</td>
<td>57.05±1.94</td>
<td>63.94±2.02</td>
<td>F=1.50</td>
<td>F=0.271</td>
<td>F=1.11</td>
</tr>
<tr>
<td>(nM/g Hb)</td>
<td>Control</td>
<td>57.68±3.53</td>
<td>62.37±1.88</td>
<td>58.60±2.31</td>
<td>P&lt;0.229</td>
<td>P&lt;0.604</td>
<td>P&lt;0.320</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=-0.575</td>
<td>t=1.96</td>
<td>t=-1.74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.567</td>
<td>P&lt;0.053*</td>
<td>P&lt;0.086</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>Supplemented</td>
<td>64.41±5.42</td>
<td>63.84±2.43</td>
<td>68.88±2.64</td>
<td>F=0.153</td>
<td>F=1.61</td>
<td>F=1.50</td>
</tr>
<tr>
<td>(nM/g Hb)</td>
<td>Control</td>
<td>63.11±4.53</td>
<td>60.65±2.50</td>
<td>60.95±2.43</td>
<td>P&lt;0.859</td>
<td>P&lt;0.211</td>
<td>P&lt;0.231</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=-0.180</td>
<td>t=-0.918</td>
<td>t=-2.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.858</td>
<td>P&lt;0.363</td>
<td>P&lt;0.032*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One way ANOVA

Figures 16, 17 and 18 demonstrate the trend of GSH alterations in three time points among the three age subgroups. There was a significant difference in supplemented group compared with control only in second time and third time point among 65-69 and 70-75 years, respectively.
**Figure 16.** Mean and SEM of GSH (nM/g Hb) in the two studied groups among subjects aged 60-64 years at three time points

**Figure 17.** Mean and SEM of GSH (nM/g Hb) in the two studied groups among subjects aged 65-69 years at three time points

**Figure 18.** Mean and SEM of GSH (nM/g Hb) in the two studied groups among subjects aged 70-75 years at three time points
5.2.4. 8-Hydroxydeoxyguanosine (8-OHdG)

The result of within groups and comparison between groups by repeated measure ANOVA for the 8-OHdG is presented in table 5.2.4.1. The test showed the difference within each group was significant (P<0.001). Comparison between supplemented and control group showed no significant difference (P<0.40).

**Table 5.2.4.1. Results of within and between groups comparisons for the 8-hydroxydeoxyguanosine (8-OHdG) based on repeated measure ANOVA**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-hydroxydeoxyguanosine (8-OHdG) (Pg/ml)</td>
<td>Supplemented</td>
<td>0.129±0.007</td>
<td>0.097±0.003</td>
<td>0.082±0.003</td>
<td>F=31.53</td>
<td>P&lt;0.001***</td>
<td>F=0.976</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.134±0.010</td>
<td>0.097±0.003</td>
<td>0.092±0.007</td>
<td>F=0.710</td>
<td>P&lt;0.401</td>
<td>P&lt;0.350</td>
</tr>
<tr>
<td>t-value</td>
<td>0.359</td>
<td>0.019</td>
<td>1.329</td>
<td>P&lt;0.72</td>
<td>P&lt;0.98</td>
<td>P&lt;0.18</td>
<td></td>
</tr>
</tbody>
</table>
| p-value | 0.72 | 0.98 | 0.18 | ** Repeated measure ANOVA**

Figure 19 as expected shows that there is a descending trend in supplemented and control group from baseline till the end of intervention but did not show any significant difference among the two groups throughout the study.

**Figure 19.** Mean and SEM of 8-OHdG (Pg/ml) in the two studied groups at three time points
As shown in table 5.2.4.2, by conducting the repeated measure ANOVA, the difference within each group (supplemented and control) was significant in the three age subgroups (P<0.001, P<0.003). Comparison between groups and interaction of time and treatment in age group of 60-64 years were statistically significant while there were not statistically significant for other age subgroups. One way ANOVA shows significantly difference between mean value of the 8-OHdG in supplemented and control for the age group of 60-64 years at baseline (0.117±0.010 VS.0.148±0.011 respectively).

**Table 5.2.4.2. Results of within and between groups comparisons for 8-hydroxydeoxyguanosine (8-OHdG) among three age groups based on repeated measure ANOVA**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supplemented</td>
<td>0.117±0.010</td>
<td>0.095±0.003</td>
<td>0.086±0.005</td>
<td>F=13.08</td>
<td>F=7.07</td>
<td>F=13.08</td>
</tr>
<tr>
<td>8-OHdG (Pg/ml) (60-64)</td>
<td>Control</td>
<td>0.148±0.011</td>
<td>0.091±0.003</td>
<td>0.089±0.004</td>
<td>P&lt;0.001</td>
<td>P&lt;0.009</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=2.07</td>
<td>t=0.737</td>
<td>t=0.518</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.041*</td>
<td>P&lt;0.464</td>
<td>P&lt;0.606</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supplemented</td>
<td>0.141±0.014</td>
<td>0.099±0.004</td>
<td>0.081±0.005</td>
<td>F=13.2</td>
<td>F=0.348</td>
<td>F=0.119</td>
</tr>
<tr>
<td>8-OHdG (Pg/ml) (65-69)</td>
<td>Control</td>
<td>0.136±0.024</td>
<td>0.100±0.006</td>
<td>0.103±0.020</td>
<td>P&lt;0.001</td>
<td>P&lt;0.557</td>
<td>P&lt;0.797</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=0.268</td>
<td>t=0.83</td>
<td>t=1.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.789</td>
<td>P&lt;0.934</td>
<td>P&lt;0.281</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supplemented</td>
<td>0.132±0.012</td>
<td>0.095±0.008</td>
<td>0.075±0.004</td>
<td>F=6.54</td>
<td>F=0.757</td>
<td>F=1.02</td>
</tr>
<tr>
<td>8-OHdG (Pg/ml) (70-75)</td>
<td>Control</td>
<td>0.109±0.011</td>
<td>0.101±0.007</td>
<td>0.080±0.005</td>
<td>P&lt;0.003</td>
<td>P&lt;0.389</td>
<td>P&lt;0.350</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=1.42</td>
<td>t=0.513</td>
<td>t=0.636</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.161</td>
<td>P&lt;0.610</td>
<td>P&lt;0.528</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One way ANOVA  ** Repeated measure ANOVA

Figures 20, 21 and 22 also show the trend of 8-OHdG level in three time points in terms of the three age subgroups.
Figure 20. Mean and SEM of 8-OHdG (Pg/ml) in the two studied groups among subjects aged 60-64 years at three time points.

Figure 21. Mean and SEM of 8-OHdG (Pg/ml) in the two studied groups among subjects aged 65-69 years at three time points.

Figure 22. Mean and SEM of 8-OHdG (Pg/ml) in the two studied groups among subjects aged 70-75 years at three time points.
5.3 Mini-Mental State Examination (MMSE) Score

In table 5.3.1, after controlling for potential covariates, including Age, sex, BMI, blood pressure, educational levels and dietary antioxidants intake, repeated measure ANOVA revealed that differences within supplemented and control group was significant (P<0.001). Comparison between groups indicated that there was no significant difference between supplemented and control group (P<0.88). Also Multivariate test of time × treatment interaction (Wilks’λ) indicated that this interaction for MMSE score was not statistically significant.

None of mean value of MMSE score in six\textsuperscript{th} and twelf\textsuperscript{th} time points between supplemented and control groups (In six\textsuperscript{th} month: supplemented VS. control 25.88±0.17 VS. 25.86±0.18 and in twelf\textsuperscript{th} month 26.8±0.17 VS. 26.59±0.18) were significant.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini Mental State Examination (MMSE) score</td>
<td>Supplemented</td>
<td>24.59±0.12</td>
<td>25.88±0.17</td>
<td>26.82±0.17</td>
<td>F=91.08</td>
<td>F=0.293</td>
<td>F=0.237</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>24.45±0.11</td>
<td>25.86±0.18</td>
<td>26.59±0.18</td>
<td><strong>P&lt;0.001</strong></td>
<td><strong>P&lt;0.589</strong></td>
<td><strong>P&lt;0.78</strong></td>
</tr>
<tr>
<td>t-value</td>
<td></td>
<td>t=1.49</td>
<td>t=1.69</td>
<td>t=0.821</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>P&lt;0.14</td>
<td>P&lt;0.091</td>
<td>P&lt;0.413</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Repeated measure ANOVA
Figure 23 shows in spite of insignificant improving among supplemented group compared with the control; there was no significant difference between them. The differences within supplemented and control group from baseline to second and third time were significant.

![Graph showing MMSE score](image)

**Figure 23.** Mean and SEM of MMSE score in the two studied groups at three time points
* significantly different from 0 and second time point (Repeated measure ANOVA)
** Significantly different from 0 and third time point (Repeated measure ANOVA)

Results of repeated measure ANOVA based on MMSE score, within and between both supplemented and control in three age groups are presented in table 5.3.1.2. Similar to table 5.3.1, this test indicates that the difference within subject variable was significant (P<0.001). There was no difference significantly between groups. Also, the interaction between time and treatment was not significant. None of the mean values of MMSE scores between supplemented and control groups were significant.
Table 5.3.2. Results of within and between groups comparisons for the Mini Mental State Examination (MMSE) among three age groups based on repeated measure ANOVA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Period 0</th>
<th>Period 6</th>
<th>Period 12</th>
<th>time</th>
<th>treatment</th>
<th>time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSE Score (60-64 years)</td>
<td>Supplemented</td>
<td>24.9±0.15</td>
<td>26.0±0.23</td>
<td>27.3±0.27</td>
<td>F=28.7</td>
<td>F=1.22</td>
<td>F=1.16</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>24.5±0.14</td>
<td>26.0±0.24</td>
<td>26.5±0.22</td>
<td>P&lt;0.001**</td>
<td>P&lt;0.273</td>
<td>P&lt;0.319</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=0.515</td>
<td>t=0.815</td>
<td>t=-0.674</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.608</td>
<td>P&lt;0.417</td>
<td>P&lt;0.502</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMSE Score (65-69 years)</td>
<td>Supplemented</td>
<td>24.2±0.23</td>
<td>25.6±0.30</td>
<td>26.2±0.29</td>
<td>F=37.9</td>
<td>F=0.340</td>
<td>F=0.654</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>24.0±0.25</td>
<td>26.1±0.40</td>
<td>26.6±0.38</td>
<td>P&lt;0.001**</td>
<td>P&lt;0.562</td>
<td>P&lt;0.522</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=-0.71</td>
<td>t=0.892</td>
<td>t=0.154</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.944</td>
<td>P&lt;0.376</td>
<td>P&lt;0.061</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMSE Score (70-75 years)</td>
<td>Supplemented</td>
<td>24.4±0.22</td>
<td>25.8±0.38</td>
<td>26.9±0.32</td>
<td>F=30.56</td>
<td>F=0.713</td>
<td>F=0.766</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>24.8±0.24</td>
<td>25.6±0.37</td>
<td>26.8±0.34</td>
<td>P&lt;0.001**</td>
<td>P&lt;0.404</td>
<td>P&lt;0.472</td>
</tr>
<tr>
<td></td>
<td>t-value</td>
<td>t=1.464</td>
<td>t=0.927</td>
<td>t=1.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>P&lt;0.148</td>
<td>P&lt;0.357</td>
<td>P&lt;0.253</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Repeated measure ANOVA

Figures 24, 25 and 26 show the alterations in MMSE scores in three age subgroups. This trend in supplemented group among 60-64 years was partially desirable in twelfth month but was not significant.
Figure 24. Mean and SEM of MMSE score in the two studied groups among subjects aged 60-64 years at three time points

Figure 25. Mean and SEM of MMSE score in the two studied groups among subjects aged 65-69 years at three time points

Figure 26. Mean and SEM of MMSE score in the two studied groups among subjects aged 70-75 years at three time points
5.4. Dietary intakes of subjects

In order to find the differences between the supplemented and control group for the dietary antioxidants intake, data from three-day dietary record for intakes of vitamin C, vitamin E, total carotene, selenium and zinc were analyzed.

The means and standard error of these antioxidants for every two months are presented in table 5.4.1. The independent t-test did not show any statistically significant differences among the two groups for intake of vitamin E, total carotene, selenium and zinc except for vitamin C in second time (P<0.029) during the study.

Table 5.4.1. Means and standard error of dietary antioxidants intake based on 3-day dietary record every two month for studied subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>First time Mean± SE</th>
<th>Second time Mean± SE</th>
<th>Third time Mean± SE</th>
<th>Fourth time Mean± SE</th>
<th>Fifth time Mean± SE</th>
<th>Sixth time Mean± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (mg)</td>
<td>supplemented</td>
<td>12.0±0.9</td>
<td>12.2±1.0</td>
<td>10.7±0.4</td>
<td>11.9±0.9</td>
<td>10.0±0.5</td>
<td>10.4±0.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>11.6±0.8</td>
<td>10.0±0.4</td>
<td>10.4±0.4</td>
<td>10.2±0.4</td>
<td>10.6±0.6</td>
<td>10.2±0.4</td>
</tr>
<tr>
<td>P-value</td>
<td>0.357</td>
<td>0.134</td>
<td>0.672</td>
<td>0.625</td>
<td>0.433</td>
<td>0.134</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>supplemented</td>
<td>90.7±6.4</td>
<td>97.0±6.5</td>
<td>92.6±5.9</td>
<td>118.4±20.1</td>
<td>103.5±6.2</td>
<td>107±5.6</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>98.6±5.8</td>
<td>110.7±6.4</td>
<td>99.9±5.9</td>
<td>108.4±6.2</td>
<td>117.6±6.8</td>
<td>109±5.4</td>
</tr>
<tr>
<td>P-value</td>
<td>0.729</td>
<td>0.029*</td>
<td>0.376</td>
<td>0.066</td>
<td>0.126</td>
<td>0.779</td>
<td></td>
</tr>
<tr>
<td>Total Carotene (µg)</td>
<td>supplemented</td>
<td>759±63.6</td>
<td>668±62.1</td>
<td>575±47.6</td>
<td>593±46.5</td>
<td>594±55.0</td>
<td>502±43.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>687±54.2</td>
<td>653±59.1</td>
<td>647±52.5</td>
<td>589±48.6</td>
<td>566±46.5</td>
<td>540±42.5</td>
</tr>
<tr>
<td>P-value</td>
<td>0.388</td>
<td>0.866</td>
<td>0.306</td>
<td>0.960</td>
<td>0.698</td>
<td>0.525</td>
<td></td>
</tr>
<tr>
<td>Selenium (µg)</td>
<td>supplemented</td>
<td>69.3±4.1</td>
<td>75.6±5.0</td>
<td>78.0±6.0</td>
<td>84.7±6.1</td>
<td>74.6±6.0</td>
<td>70.2±4.1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>67.7±4.2</td>
<td>66.9±4.3</td>
<td>72.0±4.8</td>
<td>70.0±4.7</td>
<td>66.3±4.9</td>
<td>74.1±4.9</td>
</tr>
<tr>
<td>P-value</td>
<td>0.787</td>
<td>0.188</td>
<td>0.415</td>
<td>0.550</td>
<td>0.281</td>
<td>0.552</td>
<td></td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>supplemented</td>
<td>9.2±0.3</td>
<td>8.9±0.4</td>
<td>8.7±0.3</td>
<td>9.6±0.7</td>
<td>8.4±0.3</td>
<td>8.6±0.3</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9.1±0.4</td>
<td>8.6±0.3</td>
<td>9.6±0.7</td>
<td>8.7±0.3</td>
<td>8.4±0.3</td>
<td>8.9±0.3</td>
</tr>
<tr>
<td>P-value</td>
<td>0.781</td>
<td>0.554</td>
<td>0.233</td>
<td>0.199</td>
<td>0.942</td>
<td>0.345</td>
<td></td>
</tr>
</tbody>
</table>

* independent t-test

Table 5.4.2 shows the mean and SE of dietary antioxidants intake based on 3-day food record in three age subgroups. The independent t-test did not show any
difference between supplemented and control groups in three age subgroups during the study for Vitamin E, Vitamin C, Total Carotene, Selenium and Zinc.

**Table 5.4.2.** Means and standard error of dietary antioxidants intake based on 3-day dietary record for studied subjects among three age groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Age group</th>
<th>Groups</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Supplemented</td>
<td>Control</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>60-64</td>
<td>105.8±5.9</td>
<td>109.5±7.16</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>109.4±9.18</td>
<td>107.4±7.5</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>99.2±9.4</td>
<td>104.3±5.8</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>60-64</td>
<td>12.5±1.2</td>
<td>10.7±0.47</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>10.3±0.36</td>
<td>10.0±0.38</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>12.5±1.6</td>
<td>10.8±0.68</td>
</tr>
<tr>
<td>Total Carotene (µg)</td>
<td>60-64</td>
<td>705.6±673</td>
<td>790.2±87.4</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>716.0±83.4</td>
<td>679.7±66.0</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>639.3±68.3</td>
<td>689.8±76.0</td>
</tr>
<tr>
<td>Selenium (µg)</td>
<td>60-64</td>
<td>77.3±5.4</td>
<td>72.8±7.6</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>74.6±7.2</td>
<td>73.7±5.5</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>77.9±7.6</td>
<td>73.2±12.8</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>60-64</td>
<td>11.3±1.2</td>
<td>8.7±3.4</td>
</tr>
<tr>
<td></td>
<td>65-69</td>
<td>36.8±19.1</td>
<td>9.2±0.49</td>
</tr>
<tr>
<td></td>
<td>70-75</td>
<td>9.1±0.61</td>
<td>8.5±0.35</td>
</tr>
</tbody>
</table>
5.5. The major findings

At baseline, the total number of subjects who registered in this study was 761. After evaluation by MMSE questionnaire, 296 were found to have MCI. Finally, 256 subjects remained until the end of the study.

A number of variables which act as confounding factors such as age, weight, height, BMI, blood pressure, educational attainment, dementia among close relative, marital status, household size, aerobic exercise and intake of antioxidants were not showed significant differences between supplemented and control groups.

The repeated measure ANOVA indicated that the difference within supplemented and control groups for MDA, TAC and 8-OHdG either overall or based on age subgroups was significant (P <0.001). There was no significant difference within both groups for GSH. Mean values of MDA in supplemented group at second (2.37±0.055, P<0.02) and third time point (1.75±0.067, P<0.001) were significantly different compared with the control (2.54±0.055 and 2.17±0.089) respectively. Mean values of the GSH (P <0.02) and TAC (P < 0.001) showed significant difference between supplemented and control in 12th month of intervention. Comparison between supplemented and control group for 8-OHdG level showed no significant differences (P< 0.40). Interaction of time × treatment (Wilks’λ) indicated that this interaction was statistically significant only for TAC.

After controlling of potential covariates, analysis of the MMSE scores showed a significant increasing trend within both supplemented and control groups throughout this study (P<0.001), but no significant difference between these groups were found (P< 0.88). None of mean value of MMSE score in sixth month between supplemented and control groups (25.88±0.17 VS. 25.86±0.18) and twelfth month between both of the groups (26.82±0.17 VS. 26.59±0.18) were significant.
6. DISCUSSION

6.1. Characteristics of the participants

This study was conducted to investigate the effect of supplementation with antioxidant vitamins C and E on Mild Cognitive Impairment among elderly people in Isfahan, Iran.

Naturally, along with antioxidants themselves, a wide variety of potential confounding factors are expected to influence the cognitive performance. A comprehensive review of literature provides a long list of such factors (Maxwell et al, 2005; Lloret et al, 2009). It has been proved that a number of confounding factors such as age, weight, height, BMI, blood pressure, educational attainment and dementia among close relative, marital status, household size, aerobic exercise and intake of antioxidants may influence the results of the current study through one of the following mechanism or both:

1- Direct influence on cognitive performance
2- Indirect influence on stress oxidative parameters

Brief descriptions of some of the important factors are mentioned subsequently:

6.1.1. BMI (Body Mass Index)

In the present study, control and supplemented groups with mean BMI of 26.9 and 26.3 kg/m² respectively have been categorized as groups with overweight. Obesity can act as a confounding factor. Being obese may increase risk for MCI and Alzheimer’s disease and may be associated with a great level of functional impairment (Elias et al, 2003; Mrak et al, 2009; Ho et al, 2010).

Means of BMI among subjects before and after grouping for age did not show any significant difference between supplemented and control at the baseline of the study.

6.1.2. Hypertension

Several studies point to the detrimental effect of hypertension on cognition performance. Although, the underlining mechanism(s) remain unclear, some
pathological changes, including white matter hyper-intensities on MRI or lacunar brain infarcts may contribute to such effect (Kalaria RN, 2000; Vermeer et al, 2003). Hypertension may indirectly affect cognition by influencing total antioxidant status (Subash et al, 2010). A case-control study investigating Iranian people, suggested that history of hypertension was most prevalent in AD group (Foroughan et al, 2008).

In our trial, no significant difference was observed in supplemented and control groups regarding systolic and diastolic blood pressure. It is worth mentioning that potential differences were investigated in total study sample as well as age subpopulations.

### 6.1.3. Educational attainment

Numerous studies indicate that cognitive performance of illiterates and low educated people is poorer in comparison with people higher educational attainments. In the same vein, it has been proved that screening tests such as the Mini-Mental State Examination is positively associated with schooling (Dozzi Brucki et al, 2011, de Brito-Marques and Cabral-Filho, 2004).

Overall, no statistical significant difference was observed between supplemented and control groups regarding educational attainment level. After dividing participants in to age groups, a significant difference was observed among participants in age group of 70-75 years regarding secondary education level (P<0.029). No illiterate participant was included in the current study.

### 6.1.4. Intake of antioxidants

Dietary intakes as a potential confounding factor must be considered in the current study and similar studies (Ishihara et al, 2003).

During the current study, 3-day dietary record forms were completed for each participant by a trained nutritionist every two months. Throughout the study, no significant difference was observed between supplemented and control group regarding antioxidants intake including vitamin E, Total Carotene, Selenium and Zinc except for vitamin C in second time investigation (P<0029).
Parameters of potential interest related to oxidative stress, such as smoking habits, alcohol addiction, severe coronary heart disease, significant neurological diseases and any of diseases that interfere with the antioxidant’s absorption were among exclusion criteria of this study.

In summary, it is optimal to exclude or control confounding variables. Almost, we found no significant differences between supplemented and control groups regarding weight, height, BMI, blood pressure, educational attainment, dementia among close relative, marital status, household size, aerobic exercise and intake of antioxidants as potential confounding factors in all participants as a whole, and after dividing them into age groups.

6.2. The effect of antioxidant supplementation on redox status indicators

A number of studies have shown that there is widespread oxidation of all three types of macromolecules such as lipids, proteins, and nucleic acids in MCI and AD (Mariani et al, 2005; Pratico et al, 2002; Markerbery et al, 1999; Kontush et al, 2001).

A comprehensive evaluation of oxidative stress requires determination of a large number of biomarkers, although this would be time consuming, expensive and in some cases technically difficult.

In the present study we measured some of the popular biomarkers including:
1- MDA (a non-lipophilic peroxidation product of polyunsaturated fatty Acids)
2- GSH (an oxidative sensitive molecule),
3- TAC (an estimation of overall body antioxidant capacity)
4- 8-OHdG (intermediate product of nucleic acid modification).

In our trial, vitamin E and C supplementation resulted in significant positive alterations in these indicators of redox status except 8-OHdG.
6.2.1. The effect of antioxidant supplementation on Malondialdehyde (MDA)

Lipid peroxidation is one of the consequences of oxidative damage. Several studies have indicated that individuals with MCI have increased level of lipid peroxidation and its products in the urine, plasma, CSF and brain before the onset of symptomatic dementia (Pratico et al, 2002; Montine et al, 2004; Keller et al 2005; William et al, 2007; Torres et al, 2011).

In the present study, we found an increasing trend in MDA level in both groups before six\textsuperscript{th} month, which followed by a decline, in both groups in second half year of the study. This decline was significantly more prominent in supplemented group in comparison with control group in six\textsuperscript{th} and twelf\textsuperscript{th} month after intervention. After age division of the participants of the study, the changes in MDA level within and between supplemented and control groups were statistically significant in 60-64 and 70-75 age groups respectively, but not in 65-69 age group.

Few published studies exist on influence of antioxidant supplementation on different age groups of elderly individuals.

The reason for non-significant changes in age group of 65-69 was vague.

In agreement with our trial, Krajcovicová et al conducted a study to assess the lipid peroxidation in relation to vitamin C and vitamin E levels and they found a significant inverse linear correlation between malondialdehyde and natural antioxidant levels (Krajcovicová et al, 2004).

Appearance of the effect of vitamin E on MDA level among elderly people with a high oxidative stress condition has been reported to be a slow and time consuming process. For instance, a clinical trial on the effect of vitamin E on MDA was conducted. Eccentric exercise was used as a producer of ROS in young and elderly men. Investigators showed that in baseline and following vitamin E supplementation (1000 IU) for 12 weeks, vitamin E did not inhibit the exercise-induced rise in MDA among the older men. (Sacheck et al, 2003).

Despite the fact that aging and MCI both are potential oxidative stress inducers in the current study (Rinaldi et al, 2003; Baldeiras et al, 2008) it seems that, similar to above
study, any detectable change in the MDA level may need its sufficient time (Goi et al, 2005). The discrepancy between our trial and Sacheck et al study can be attributed to two major differences: 1- shorter period (12 weeks) of antioxidant supplementation and, 2- the use of only one antioxidant (vitamin E) in comparison with the use combined vitamin E and C in our study.

In contrast, two randomized controlled trial suggested that supplementation with vitamin C or vitamin E alone would be more effective than their combination (Huang, et al 2002; Dietrich et al, 2002).

In a study conducted by Huang et al, the effect of supplementation with vitamin C (500 mg) and/or vitamin E (400 IU) for 2 months on lipid peroxidation among 184 non-smoker participants were examined. The study showed a reduction in lipid peroxidation of 10% on the basis of the measured urinary excretion of 8-iso-PGF2α (a major isoprostane). In their study, vitamin C alone was more effective than vitamin E alone and combined vitamin E and C had no additional benefit (Huang, et al 2002).

The similarities between our trial and the study of Huang, et al were age group, type and dosage of used antioxidant and the differences were the duration of supplementation (2 months) and the type of biomarker of lipid peroxidation. Naturally, longer duration of intervention could increase the reliability of results.

According to the findings of another clinical trial, dietary vitamin E supplementation between 100 and 300 mg/d for 4 months had been apparently able to decrease plasma MDA concentrations in healthy middle-aged and elderly people. (Sun et al, 2012)

The results of the current study are congruent with the results of the above study. The age group and the amount of vitamin E given in two studies were approximately the same.

In summary, data from effects of antioxidants on lipid peroxidation have almost been inconsistent. Part of the reason for this inconclusive result may be due to different participants with different features, different antioxidants used (vitamin C and vitamin E in combination or alone), different dosages and different time periods.
6.2.2. The effect of antioxidant supplementation on total Glutathione (GSH)

Glutathione (GSH) is the most abundant intracellular thiol antioxidant. It plays a central role in the maintenance of cellular redox level by removing free radicals generated during oxidative stress and maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms (Sathyavathi et al, 2011).

Antioxidant supplementation is naturally expected to increase the GSH level. In the current study, red blood cells GSH content was significantly higher in supplemented group at the end of intervention. After age categorization, mean differences of GSH in the second time point of the study were significant in 65-69 age groups and the third time point was significant in 69-75 age group (P < 0.05, P < 0.03).

The real cause of this delay in GSH level elevation is not clear and many environmental and genetic factors may have influenced it:

1. The depletion of the vitamin reserves in the body can cause this problem. For instance, Long-term intake of estrogen and other hormones therapy during menopause cause a depletion of vitamin C and other cellular nutrients in the body (Rath M, 2003) which could reduce the effectiveness of vitamin E to protect against oxidative stress damage in the elderly (Ryan et al, 2010).

2. Several studies indicated that oxidative stress is increasing in elderly individuals with MCI (Pratico et al, 2002; Montine et al, 2004; Keller et al 2005; William et al, 2007; Torres et al, 2011) thus, more time will be needed to display the improving of GSH level.

3. Reports show that in some aging individuals, absorption of several substances including micronutrient is reduced (Husebye and Engedal, 1992).

4. Aging is known to contribute to significant vitamin E deficiencies especially among elderly people with MCI (Kontush et al, 2001; Rinaldi et al, 2003; Baldeiras et al, 2008).
Therefore, an increase in consumption of antioxidant in patients with MCI could be helpful to fill the depleted reservoirs and after a period of time tend to be improved the redox status (Meydani M, 2001).

A double-blind clinical trial by Jain, et al examined the effect of vitamin E supplementation on glutathione concentrations in the erythrocytes of type 1 diabetic patients. They indicated that vitamin E supplementation (100 IU/day) for 3 months, significantly increased glutathione in the patients (Jain et al, 2000). This study also concluded that, erythrocytes of diabetic patients had 15% lower glutathione concentrations than healthy subjects; therefore diabetes can be seen as an oxidative stress inducer in patients (Al-Aubaidy and Jelinek, 2011). In their study GSH level was increased in a short term compared with the current study.

There are several major differences between the present study and the study of Jain, et al. One of the most important differences was the age group of participants that were aged 10 to 13 years in the study of Jain, et al. Although, participants in both studies have somehow increased oxidative stress but young people have been reported to react better to antioxidant supplementation than old (Filaire et al, 2011). As mentioned, both aging (Giblin FJ, 2000; Spiteller G, 2001) and MCI (William et al, 2007; Torres et al, 2011) in our trial are prone to increase the oxidative stress.

A major limitation of Jain et al’s study was the sample size. 12 samples are too small to make any reliable judgment about the results. The sample of the current study included 265 participants.

The reports about the effect of antioxidants on GSH level have been inconsistent. While, in a clinical trial among people with non-insulin-dependent diabetes mellitus, 400 mg/day vitamin E for one month increased the GSH level (Sharma et al, 2000), another study indicated that the effect of vitamin E (280 mg/day) for 10 weeks reduced erythrocyte total glutathione concentration (Brown et al, 1996).

It seems that other unknown factors may influence non-significantly increase of glutathione in the first 6 months of our trial.
In summary, there are long list of factors which may influence the GSH level. A major difference between the current trial and previous studies is the duration of supplementation. A longer period of supplementation may be required to make judgment about the effect of supplementation on redox status.

### 6.2.3. The effect of antioxidant supplementation on Total Antioxidant Capacity (TAC)

Measurement of Total Antioxidant Capacity (TAC) is a known good approach to evaluate the bioavailability of antioxidants (Nälsén et al, 2006). In the present trial, there was an increasing trend in TAC, which reached to a statistically significant level in antioxidant received group at the end of study. Age classification did not change this pattern and in all age groups increasing of TAC was observed at the end of intervention.

TAC level in the body could be modified by the diet. As mentioned, in our study, dietary antioxidants intake, as an important confounding factor, was assessed by the 3-day food records, and showed not to be statistically different in supplemented and control groups.

In a study by Nälsén et al, a higher dietary intake of ascorbic acid, tocopherols and β-carotene, among men with a mean age of 60 for one year, was indicated a higher plasma TAC (Nälsén et al, 2006). Although, the mentioned study and the current trial have indicated similar results, there are some differences between them. In the mentioned study, daily antioxidants intake was assessed through 24-hour recall method while in the current study the amount of taking antioxidant is predetermined. The 24-hour recall is prone to errors because it dependents on the memory of investigated subject and the responders may have forgotten some food items (Biro G, 2001). In their findings, in addition to vitamin E and C, β-carotene was also calculated.

Another limitation of their study was that blood samples were collected only one time at the end of their study while in the current study, TAC was measured in three times throughout the trial.
Another clinical trials study among elderly people with diabetes mellitus performed to determine the effect of two consecutive moderate and high doses of vitamin C and E on TAC. They used vitamins C (500 mg) and E (400 IU) for 4 weeks. Following a 4 weeks, the patients had a further 4 weeks of supplementation with a higher dose combination of vitamins C (1000 mg) and E (800 IU). TAC was significantly increased after both doses (Nuttall and Martin, 2002).

In the current study, TAC level increased only at 12th month of supplementation, but in above trial TAC level enhanced after two months. This discrepancy between two studies can be due to higher dosage of antioxidant used in their study. Another problematic point is their sample size. In their study, a total of 9 participants are too small to sufficiently provide accurate results.

In another experiment, Supplementation with an enriched drink contained vitamin C (225 mg); vitamin E (55 mg) and selenium accompanied with macronutrients two times a day, for six months were examined among frail elderly participants. Their results showed a significant increasing in TAC among supplemented group. (Wouters-Wesseling et al, 2003). In their study, frail elderly who are at risk of nutritional deficiencies were selected. It would be possible that frail elderly absorb antioxidants more extent than healthy elderly. Another point is using selenium along with vitamin E and C as a potent antioxidant. Using other macronutrient may also improve their antioxidant capacity during their study (6 months).

In summary, there is a positive association between antioxidants intake and increasing the total antioxidant capacity, however, some factors including duration of supplementation, dosage and type of antioxidant, can influence this relationship.

6.2.4. The effect of antioxidant supplementation on 8-hydroxydeoxyguanosine (8-OHdG)

In the present trial, 8-OHdG as a marker of oxidative damage to DNA was measured in three time points. The obtained results showed that there was an apparent descending trend in serum 8-OHdG concentration, in both supplemented and control
groups, from baseline to the end of intervention but this decline did not reach to a statistically significant level. After age classification, the same results were observed. Although, it is expected that antioxidant vitamins inhibit the oxidative DNA damage, studies on human subjects regarding the effect of antioxidant on 8-OHdG level in different conditions have yielded inconsistent results (Igishi et al, 2003).

Despite the fact that combined vitamin C and vitamin E had apparent effect on MDA, TAC and GSH in present trial, no detectable effect on 8-OHdG was observed. This may be due to:

- The observed result may be attributable to environmental and/or genetic factor(s) that were not examined or checked out in this trial.
- Likely, one year intervention period may not be enough to reveal the possible effects of vitamin C and E supplementation on 8-OHdG levels thus, prolongation of the intervention period, may meet our expectations.
- Using an appropriate type and a larger dose of antioxidant may reduce the 8-OHdG level in serum.
- Although, aging and MCI both can cause oxidative stress, it seems that their effects on nucleic acids is not strong enough to increase 8-OHdG level upper than the base markedly. This may explain why antioxidants have not shown any effect on 8-OHdG.

Our result is consistent with a previous placebo-controlled trial in which two months supplementation with vitamins C and E on non-smoking adults did not yield any significant effect on urinary excretion of 8-OHdG (Huang et al, 2000).

The measuring method of 8-OHdG and duration of supplementation in two studies were different but the dose of antioxidants and sample sizes were similar. Another study measured serum level of 8-OHdG among 15 healthy smokers and 5 healthy non-smokers as control. Effects of antioxidants including vitamin E, β-carotene, vitamin C and red ginseng in 4 subgroups for 4 weeks were investigated. Their finding revealed that the levels of 8-OHdG were gradually decreased in serum of smokers using each antioxidant but this decline is more prominent in vitamin E group (Lee et al, 1998).
The most important limitation of the mentioned study was the size of sample. Three participants in each group are too small to achieve a decisive conclusion. Another point is that they ignored some of the confounding factors such as: assessment of dietary antioxidant intake (Huang et al, 2000), BMI (Al-Aubaidy and Jelinek, 2011), hypertension (Subash et al, 2010) and etc.

Another factor that may have influenced the result is the difference between ages in the current study and their study. Participants in their study were all male students aged of 19 to 31 years. As stated before, young people react better to antioxidant supplementation than old (Filaire et al, 2011). Also, in the present study, all volunteers were nonsmoker compared with the above study. Cigarette smoke causes significant oxidative stress (Kinnula V, 2005) and in smoker people with severe oxidative stress condition, the antioxidants effects seem to be more prominent.

Effect of vitamin C on 8-OHdG level among smoking abstention group has been shown in another clinical trial. The result suggested that vitamin C (2g/d) for 4 weeks could not decrease 8-OHdG level significantly (Inoue et al, 2003). Their findings were also similar to our result in spite of the fact that their pill taking duration was too short and the dose of vitamin C was too high (2g/d).

In summary, previous studies about the effect of antioxidants on 8-OHdG have yielded contradictory results. Most of the former studies have involved the smoker volunteer with a small size of samples and thus the comparison between their results with those of current study would be inaccurate.

### 6.3. The effect of antioxidant supplementation on cognitive performance

In our study, one year supplementation with combined vitamin E and C, in spite of improvement in oxidative stress markers, was not seen to have remarkable effect on cognitive performance.
These observations are consistent with some of the previous studies. In contrast, they are not in line with the some other observations. While numerous epidemiological or clinical studies have proposed and explained a link between antioxidant intake and a reduced incidence of dementia or cognitive decline in elderly populations (Masaki et al, 2000; Morris et al, 2002; Grodstein et al, 2003; Engellart et al, 2002; Zandi et al, 2004), a noticeable other studies failed to reveal such effect (Isaac et al, 2008; Sano et al, 1997; Petersen et al, 2005).

The real cause(s) of this inconsistency among various experiments is not clear, but a long list of underlining factors could be listed:

6.3.1. Duration of intervention

Duration of intervention is a major difference between the current study and some of the similar studies and may be one of the causes of difference in responsiveness to antioxidants supplementation.

Duration diversity in antioxidants supplementation in various studies regarding cognitive performance has led to contradictory result. For instance, antioxidants supplementation for more than 10 years (Grodstein et al, 2003), more than 7 years (Wengreen et al, 2007) about 2 years (Sano et al, 1997) yielded positive results while some others with nearly similar duration failed to do so (Kang et al, 2006 with 10 years) (Petersen et al, 2005 with 3 years) (Luchsinger et al, 2003 with 4 years) (Maxwell et al, 2005 with 5 years).

Although, Grodstein et al, argued that prolongation of antioxidant administration will increase the likelihood of beneficial effect on cognition, other studies with relatively long term treatment (about 10 years) (Kang et al, 2006), have not achieved the positive results. Therefore, it doesn’t seem that supplementation duration alone to be a remarkable factor in effectiveness of antioxidants.

In our trial we selected one year period of antioxidant supplementation, and from our view point, this is an average duration regarding the previous similar studies. This duration seems to be an efficient time to make judgment regarding modification of cognitive performance and also enough time to monitor oxidative stress biomarkers.
Supplementation duration must be noted along with other influencing factors especially the dose of antioxidants.

**6.3.2. Dose of antioxidants**

An important factor which naturally has remarkable influence on outcomes of the current and similar studies is the dosage of administrated antioxidants. In our study, supplemented group consumed 300 mg of vitamin E (α-tocopheryle Acetate) and 400 mg vitamin C(Ascrbic acid) per day. Vitamin E is considered relatively safe compared to other fat-soluble vitamins. An increase in mortality at high dosages of vitamin E is biologically plausible. High-dosage (> or =400 IU/d) of vitamin E supplements may increase all-cause mortality and should be avoided (Miller et al, 2005; Lock and loblaw, 2005). Evidence from the selenium and vitamin E in Cancer Prevention Trial suggests that daily supplementation with 400 IU vitamin E in healthy men may significantly increase the risk of prostate cancer (Klein et al, 2011). However, dosage and duration of administrated antioxidants should be assessed in the same time. According to a study, consumption of 800 IU (727 mg) of vitamin E for 4 months did not adversely affect healthy elderly persons (Meydani, et al, 1998). Consuming of high dose of vitamin C supplementation has been reported to be associated with higher risk of age-related cataract (Rautiainen et al, 2010) or hyperoxaluric nephropathy and progressive renal failure (Rathi et al, 2007). According to the some literature, the authors advised 1 g daily intake of vitamin C supplementation to ensure an optimal allowance of vitamin C (Deruelle and Baron, 2008).

Previous clinical trial studies on the effect of antioxidants on AD or MCI using a different dose of antioxidant indicated contradictory results. These includes the studies with negative result (kang et al, 2006 with 600 IU vitamin E alone) ( Petersen et al, 2005 with 2000 IU vitamin E + 10 mg donepezil)( Galasko et al, 2012 with 800 IU/d of vitamin E + 500 mg/d of vitamin C + 900 mg/d of α-lipoic acid + 400 mg of coenzyme Q)and those with positive result (Morris et al, 1998 with 400 IU vitamin E, 500 mg vitamin C) (Sano et al,1997 with 2000 IU Vitamin E+ 10 mg Selegiline).
Regarding above studies, some trials that have been categorized as high dose user of antioxidants, were not able to obtain positive result while some studies have been revealed positive results by using lower doses.

According to a meta-analysis survey (Williams et al, 2010) in most studies which obtained the positive results, the dose of antioxidant were unknown and calculated from self-report responses. Therefore, dosage of antioxidants is not the solely determinant of the outcome and must be noticed along with other effective variables.

### 6.3.3. Using vitamin E and C in combination

There was an important rationale for us to choose both of these two antioxidants for the supplementation. Ascorbate, the major hydrophilic antioxidant, and α-tocopherol the main form of vitamin E and the major lipophilic antioxidant tended to be lower in serum (Kontush et al, 2001; Baldeiras et al, 2008) and Cerebrospinal fluid (CSF) of MCI and AD patients (Schippling et al, 1999; Schippling et al, 2000; Kontush et al, 2001).

It is most likely that a single antioxidant may not provide sufficient neuro-protection in this disorder. Animal study also supports the additional benefits with the combination (Sato et al, 1993). Kontush et al also revealed that supplementation with vitamin E + C has raised α-tocopherol level in CSF (45%) than plasma (35%) (Kontush et al, 2001). Antioxidants used in combination promote defense mechanisms against oxidation and may be more efficient to neutralize the oxidative component of the pathogenesis of AD than each of these factors alone (Yeum et al, 2009; Markesbery and Lovell, 2007; Stocker R, 1994; Wengreen et al, 2007).

### 6.3.4. Type of antioxidants

The selection of proper type(s) of antioxidant(s) for a study such as ours has always been a challenge. There is a long and increasing list of antioxidant which seems to be proper candidate for studying MCI- antioxidant relation.
In our trial, combined vitamin E (α-tocopheryle Acetate) and vitamin C (Ascorbic acid) were used with a medium dose. Vitamin E has eight natural compounds, (Morris et al, 2005) among them α-tocopherol is the most biologically active form of vitamin E and the most potent antioxidant. Most studies have used this compound of vitamin E in relation to AD and MCI. Gamma-tocopherol, another form of vitamin E, has been found to be more effective in scavenging free radicals and nitrogen oxygen species; both of these are components of neurodegenerative disorders (Usoro et al, 2010; Jiang Q et al, 2003; Williamson et al, 2002). Most studies using α-tocopherol in preventing AD and managing MCI resulted in negative results. Therefore, in a recent study, authors recommended that future trials assessing vitamin E treatment in AD should not be restricted to alpha-tocopherol. (Farina et al, 2012)

Beta-Carotene is also another antioxidant which has been used by several studies in relation to MCI and AD (Stuerenburg et al, 2005; de Oliveira et al, 2012; Rinaldi et al, 2003). Beta-carotene, due to its deleterious effect on smokers (Arora et al, 2001) and because of using vitamin E as a major lipophilic antioxidant similar to beta carotene, did not use in our study.

6.3.5. Challenging with some of the important studies

Morris, et al, in their prospective study examined the association between antioxidant supplements containing vitamin E and C, and the prevention of Alzheimer’s disease. After an average follow-up period of 4.3 years, use of vitamin E or C supplements associated with reduced incidence of AD (Morris et al, 1998).

One of the important problems in the above study was to fail to consider some of the confounding variables. In other words, they have not been eliminated. For instance, AD has been reported to be associated with educational attainment (Sattler et al, 2012; Letenneur et al, 1999), body mass index and lifestyle factors such as physical activity (Scarmeas et al, 2009; Buchman et al, 2005) and older age and sex (Letenneur et al, 1999; Kukull et al, 2002). These factors have been ignored by the authors.
Grodstein et al found evidence of better cognitive performance among long-term users of combined vitamin E and C. In their study, information on use of the supplements was collected biennially via mailed questionnaires. Daily dose of the vitamins were determined by self-reported questionnaires (Grodstein et al, 2003). Their findings were opposite to our results. This may be due to:

- In their study, doses of vitamin E and C were higher and the duration of supplementation was longer than our trial.
- The dose of taking antioxidants was estimated using self-reported questionnaire and it can reduce the accuracy of data.
- No assessment of antioxidant consumption such as measurement of serum vitamin E and C levels or determination of the redox status indicators was done. These evaluations are necessary to ascertain about consumption of antioxidants (Lloret et al, 2009).

In another study, Lloret et al emphasized the necessity of determination of redox status indicators, as we did in our study. They introduced the novel concept of stratifying AD patients according to responsiveness to antioxidants. They found two distinct sub-populations of patients i.e.: respondents and non-respondents based on measures of plasma GSSG (the oxidized glutathione) and MDA. Half of the patients did not show any significant decrease in GSSG, while in others GSSG decreased with vitamin E (800 IU/d) treatment. In none of them MMSE score changed significantly. Investigators suggested that every oral supplementation study should be followed by determination of oxidative stress biomarkers (Lloret et al, 2009).

In our trial, MDA, GSH and TAC significantly improved but cognitive function was not enhanced. Although, the results between two studies are similar but a number of limitations exist in their study:

Their study was not large enough (only 33 completed the study) to detect alteration of GSSG and MDA more reliably. Also, their intervention period was short (6 months) to make any judgment about modification of cognitive performance using MMSE score.
Another weakness of Lloret et al’s study was that they confined their markers to only two; namely GSSG and MDA, and this seems barely sufficient to assure the effects of vitamin E on redox status. Especially, they didn’t assess dietary antioxidant intake which can be an important confounding factor. Bioavailability of vitamin E can be another factor among non-respondents which has been ignored in their study. The subjects suffering from mal- abortion or GI tract disorders should be excluded.

The reason of positive effect of vitamin E in reduction of the GSSG during their six months intervention may be due to consuming high dose of vitamin E (800 IU/day) as compared with our study (300 IU/day). In our study glutathione significantly increased in twelfth month of intervention.

The Advantages of the current study compared with Lloret et al’s study include the following:

1- vitamin E combination with vitamin C,
2- longer period of intervention,
3- A larger sample size,
4- Determination of four redox status indicators.
5- In our trial, several confounding variables were found to be not significantly different between supplemented and control groups, but in their study, these factors have been ignored.

On the other hand, one of the advantages of their study is using four different tools to evaluate the participant’s cognition function while in our study only one instrument was used.

Mini Mental State Examination (MMSE) which is used to evaluate of cognitive performance in the current trial seems to be quite useful in examining patients at increased risk for dementia (e.g., Mild Cognitive Impairment), particularly when age and education adjustments were implemented (Petersen et al, 2001; Folstein et al, 1975). The MMSE offers modest accuracy with the best value for diagnosis of dementia in community and primary care (Mitchell A J, 2009; Pezzotti et al, 2008; Siu Al, 1991).
Seyedian et al, also suggested that Farsi version of MMSE (F-MMSE) has acceptable validity and it is applicable in Iran (Seyedian et al, 2008). Nevertheless, the MMSE appears to have limitations as a sole screening tool in general population assessments. One of the most important limitations of the test is the fact that participants will become familiar with this test following their first evaluation and this familiarity may affect the discriminatory efficacy of this test in subsequent evaluations and may act as a confounding factor. As indicated in result section, within each supplemented and control group, MMSE score was significantly increased in each time points. It seems that participants have acquired knowledge regarding the test following their first evaluation. Therefore, simultaneously using a number of assessing tools may provide more accurate results.

In another study, results similar to our trial were found. In their double-blind experiment, subjects with MCI were randomly assigned to receive 2000 IU of vitamin E daily, 10 mg of donepezil (the most widely used cholinesterase inhibitor) or placebo for three years. There were no significant differences in the rate of progression to AD between vitamin E and placebo groups. They also noticed the changes of cognition, assessed by several tools including MMSE. Their study indicated that vitamin E, even when taken in large doses over long periods of time, was not able to improve or maintaining the MCI (Petersen et al, 2005). Despite some of the differences regarding the dose of vitamin E (2000 IU), intervention period (three years) and the sample size (769), the same results have been obtained by the two studies.

Assessing the rate of progression to AD from MCI was beyond the scope of the current study. The main reason is that MMSE did not seem to be the proper instrument for such an assessment. As mentioned above, the MMSE scores obtained by participants showed an upward trend during subsequent evaluations. Logically, this can be attributed to the practice effect that is defined as the effect of the past-experience of taking a test on taking that same test again. This may mask potential worsening of cognitive function. Although, the MMSE questionnaire is considered as
desirable tool to compare the cognition performance between the two groups, it is by no means applicable to distinguish the worsening of cognition within each group. The limitation of the Petersen et al study includes:

The investigators didn’t monitor the alteration of vitamin E level or any other stress oxidative markers in subjects.

Confounding factor, which may lead to bias in the study, including BMI, blood pressure, educational attainment, dementia among close relative, aerobic exercise and intake of antioxidants were not mentioned to be assessed in their study.

Another important point in the above study was using high dose of vitamin E during three years. As stated before, mega dose of the vitamin E may be deleterious for elderly individuals.

7. Conclusions

Epidemiological or clinical studies have not provided the ultimate answer to whether antioxidants can truly improve the cognitive performance. This may be attributable to several reasons listed in the text. In our study, one year supplementation with vitamin E and C combination resulted in positive changes in oxidative stress markers; however, there was not any remarkable effect on cognitive function, although several confounding factors have been controlled. A large number of kinetic and/or dynamic factors could be responsible for this unresponsiveness. Further studies especially with more efficient cognitive evaluation tools as well as more diverse antioxidant types may be needed to clarify the possible antioxidant-oxidative stress-cognition interactions.
Limitation of the present study

Although, the present study has yielded some preliminary results, its design is not perfect. A number of weakness need to be noted regarding the present study. The main limitations are expressed as follows:

- Using of MMSE questionnaire alone, as a most widely used cognitive test in evaluation of MCI, seems to be not sufficient for our purpose. Concomitant using of MMSE with some other tools in the same time would be more informative.
- Lack of knowledge about the best duration of intervention for researchers
- After the first MMSE exam the participants will become familiar with its questions. This familiarity may affect the discriminatory proficiency of this test in subsequent evaluations and may act as a confounding factor.

- Lack of knowledge about the best type of antioxidants and proper therapeutic dosage

Suggestion

In order to achieve clearer cut results about the possible interaction between antioxidants and cognitive function, future studies are proposed to consider these factors:

- Using of several cognition assessing tools
- Measurement of larger number of oxidative stress marker, just before and during the experiment
- Measurement of plasma concentration of administrated antioxidants

Therefore, use of multiple antioxidants, supported by measurements of several different biomarkers and determining of the antioxidant concentration of patients, is recommended.
Summary

There are inconsistent results regarding the potential role of vitamin antioxidants in improving cognition function among elderly individuals.

This study was designed as a double blind, randomized clinical trial to investigate the effect of supplementation with antioxidant vitamins C and E on Mild Cognitive Impairment (MCI) in Isfahan, Iran. Two hundred and fifty six elderly volunteers between the ages of 60 -75 years with MCI who met the criteria were randomly assigned into two supplemented and control groups. Each of these groups was further divided to three (60-65, 65-70 and 70-75) age subgroups. Randomization was performed using a stratified randomization. MCI assignment was done on the basis of 21-26 scores in MMSE.

The case group consumed 300 mg of vitamin E (Dl-α-tocopherol acetate) plus 400 mg vitamin C (Ascorbic acid) per day, for one year. Controls received placebo for the same duration.

Information on background characteristic and anthropometric measurements at baseline and food consumption was collected through Three-Day Diet Recall every two months. All cases underwent a thorough biochemical and cognition performance evaluation at three stages on days 0, 180 and 360 post interventions.

Numerous potential confounders including age, weight, height, BMI, blood pressure, educational attainment, dementia among close relative, marital status, household size, aerobic exercise and intake of antioxidants were analyzed and found generally not to be different between groups with a few exceptions.

Antioxidants supplementation for one year resulted in:

1- The difference within each supplemented and control group variable in terms of MDA, TAC and 8-OHdG in all subjects and all age subgroups was significant while in GSH there was no significant difference within each of groups.

2- Mean value of MDA revealed significant reduction at six\textsuperscript{th} and twelf\textsuperscript{th} month and elevation of TAC and GSH only in twelf\textsuperscript{th} month in supplemented group compared with the control.
About MMSE scores, After controlling for potential covariates including Age, sex, BMI, blood pressure, educational levels and dietary antioxidants intake, repeated measure ANOVA analysis of the MMSE scores showed a significant increasing trend within both supplemented and control groups throughout this study (P<0.001), but no significant difference between these groups were found (P< 0.88).

None of the mean values of MMSE scores in six\textsuperscript{th} and twelf\textsuperscript{th} time points between supplemented and control groups (In six\textsuperscript{th} month: supplemented VS. control 25.88±0.17 VS. 25.86±0.18 and in twelf\textsuperscript{th} month 26.8±0.17 VS. 26.59±0.18) were significant.

Although positive changes in the oxidative stress biomarkers was observed, based on the current study, antioxidants supplementation would not enhance cognition performance.
Zusammenfassung


Der Unterschied der MMSE-Scores war, nach Berücksichtigung der Kovarianzen Alter, Geschlecht, BMI, Blutdruck, Bildungsniveau und Einnahme von Antioxidantien, innerhalb der Gruppen (supplementierte- und Kontrollgruppen) signifikant (p<0.001), aber nicht zwischen den Gruppen: p<0.88 (nach sechs Monaten: supplementierte vs. Kontrollgruppen, 25.88 ± 0.17 vs. 25.86 ± 0.18, nach zwölf Monaten 26.8 ± 0.17 vs. 26.59 ± 0.18).

**Conclusio:** Obwohl die Parameter des oxidativen Stress durch die Supplementierung positiv beeinflusst wurden, konnte die Verabreichung der Antioxidantien Vitamin C und Vitamin E die kognitive Leistung unter den gegebenen Studienbedingungen nicht verbessert werden.
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* The survey of the nutritional status of 2-3 years old rural children and some factors affecting it in the rural areas of Kerman.

* The study of effects of nutrition education on knowledge and practice of mothers, and its relation with birth weight in squatter settlements of Kerman

* Obesity among schoolchildren and some effective factors on it in Kerman city

* Intervention program to improve healthy snack among students in Isfahan province, Iran

* Obesity among urban Thai school children (10-12 years) and its relationship to the family environment, food habits, and activity patterns in Thailand
*Nutritional status of elderly and some effective factors on malnutrition in Isfahan city.

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*Efficacy of vitamin A and vitamin A plus Riboflavin and iron status in rural primary school pupils in Kerman province

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