ASSESSMENT OF POSSIBLE ASSOCIATION BETWEEN ANTIOXIDANT LEVELS AND APOE GENE POLYMORPHISM IN DEMENTED SAUDI POPULATION

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ABSTRACT

To determine the possible associations between antioxidant serum markers and apolipoprotein E (APOE) genotypes in an elderly demented Saudi population. There were 80 subjects included in this study classified according to cognitive function as two groups control and demented patients. Antioxidant capacity and lipid peroxidation were measured using spectrophotometric analysis. APOE genotypes were determined using restriction enzyme analysis. Correlations of serum levels of antioxidant capacity and lipid peroxidation with APOE genotypes were assessed. Serum antioxidant capacity was significantly reduced in patients group in comparison to the control (p-value= 0.0125< 0.05). Also, lipid peroxidation level showed significantly higher concentration on patient compared to control group (p-value=0.0167<0.05). For APOE, three alleles (E2, E3 and E4) and four genotypes (E2/3, E3/3, E3/4 and E4/4) were identified in present study. There was a significant different between alleles and genotypes distribution in the study groups as the frequency of E4 was higher in the subjects with dementia compare toin control (p-value=0.0168<0.05) and (p-value=0.0447<0.05), respectively. However, no association was found between APOE status and serum levels of antioxidant capacity and lipid peroxidation. In conclusion, no correlation between antioxidant capacity or lipid peroxidation levels and APOE genotypes. They are independent risk factors for dementia in the Saudi population.

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1 Introduction

In recent years, healthcare services are improved that lead to increase in longevity and life expectancy. Dementia is not considered as a normal part of aging, but its prevalence become more common among an elderly population (Prince et al., 2013; Castro-Chavira et al., 2015). Dementia is an acquired cognitive impairment syndrome with slow progression that can be caused by a range of diseases and injuries to the brain as neurocognitive disorder (American Psychiatric Association, 2013; Thakur, 2015; Chen et al., 2016). It is characterized by progressive loss of cognitive and intellectual functions, especially memory and behavioral disabilities that interferes with a person’s daily life (World Health Organization, 2012; Alzheimer’s Association, 2017). Causes of dementia can vary, depending on the types of brain changes that may be taking place and there are many genetic, lifestyle, vascular risk factors including in its pathogenesis. The most common subtype of dementia is Alzheimer’s disease and other types include Vascular dementia, Lewy body dementia and Frontotemporal dementia (World Health Organization, 2012; Alzheimer’s Association, 2017). However, it is common for people to have these pathologies together as mixed dementia (Jellinger, 2007; Lee, 2011; Castro-Chavira et al., 2015).

In the case of most progressive dementia there is no known cures available (Williams et al., 2010; Lundkvist et al., 2014; Alzheimer’s Association, 2017). Though, there are multiple pharmacological and non-pharmacological treatments that have been proven to slow disease progression and treat symptoms (Rosini et al., 2014; Jedenius et al., 2015; D’Onofrio et al., 2016; Alzheimer’s Association, 2017).

The genetic aspects have been indicated to play an important role in the dementia development. The different subtypes of dementia have different structural genomics (Ferencz & Gerritsen, 2015). For example, Alzheimer’s disease has more than 200 genes that might be involved in its pathogenesis (Cacabelos, 2008). In general, only apolipoprotein E4 gene is consistent between studies as the strongest genetic risk factor linked to most common dementia subtype in various populations (Harold et al., 2009; Percy et al., 2014; Alzheimer’s Association, 2017).

The human apolipoprotein E (APOE) is a 299 amino acid glycoprotein that plays a key role in lipid transport and lipoprotein metabolism through both the vascular and nervous systems by binding to members of the low-density lipoprotein receptor family (Bu, 2009). The APOE gene is mapped to chromosome 19 which consists of four exons and three introns. The structural gene locus for APOE is polymorphic having three common different alleles APOE2 (cys112, cys158), APOE3 (cys112, arg158) and APOE4 (arg112, arg158). This amino substitution results in not only structural differences, but also physiologic differences such as their binding affinity for specific lipoprotein receptors, antioxidant properties, inflammatory responses and neuronal processes such as development and plasticity. Additionally, each of the APOE alleles is associated with differing risks of specific diseases (Mahley et al., 2006; Frieden & Garai, 2012; Liu et al., 2013).

The E2, E3 and E4 alleles have a world-wide frequency of 8.4%, 77.9% and 13.7%, respectively (Riddell et al., 2008; Holtzman et al., 2012; Kang et al., 2016). There are three homozygous (E4/4, E3/3 and E2/2) and three heterozygous (E2/4, E3/4 and E2/3) genotype.

The prevalence of APOE alleles and genotypes in Saudi population was closed to other population. A study on healthy unrelated Saudi subjects observed that the allele frequencies of APOE were 79% for E3, 15% for E4 and 6% for E2 and five genotypes were detected (E3/E3, E4/E4, E2/E3, E2/E4 and E3/E4) with prevalence as percentage 63, 2.5, 8.5,1 and 25, respectively (Awad & El-Tarras, 2011).

The APOE3 is most common and considered with normal lipid metabolism. However, APOE2 and APOE4 isoforms are related to abnormal lipid metabolism and associated with risk of many diseases.

The APOE4 has been presented more harmful effects in the brain as it associated with neuronal mitochondrial dysfunction, decrease GABAergic interneuron selectivity, greater neuronal inflammation, less efficient neuronal repair, blood brain barrier (BBB) dysfunction, Aβ accumulation, reduction in cerebral blood flow and hypoxia (Zlokovic, 2011; Leduc et al., 2011; Ringman et al., 2012; Villeneuve et al., 2014).

The frequency of the E4 allele is dramatically increased risk factor of most common form of dementia while the E2 allele being protective relative to the prevalent E3 allele (Corder et al., 1993; Dewji & Singer, 1996; Huang, 2010; Liu et al., 2013; Vos et al., 2013). Also, APOE4 carriers develop dementia 8–20 years earlier than non-carriers (Bertram & Tanzi, 2008; Verghese et al., 2011; Mahley & Huang, 2012; Panza et al., 2012).

The APOE4 has been extensively studied in major subtypes of dementia including Alzheimer’s disease, mild cognitive impairment, vascular dementia diseases, Lewy body disease and frontotemporal dementia, however most studies have failed to report associations between APOE4 and susceptibility to Parkinson’s disease and PD-associated dementia (Rubino et al., 2013; Zhou et al., 2014; Wang et al., 2014; Rohn, 2014; Bras et al., 2014; Walker et al., 2015; Yan et al., 2016; Chen et al., 2016).

On the other hand, increasing evidence demonstrates that oxidative stress causes damage to cell function with aging and it is also involved in a number of age-related neurodegenerative
disorders such as dementia (Niedzielska et al., 2016). In some circumstances the production of reactive oxygen species and reactive nitrogen species can exceed the endogenous antioxidant ability to destroy them and an oxidative imbalance occurs (Pham-Huy et al., 2008; Halliwell & Gutteridge, 2015). This event results in cellular oxidative stress and subsequent molecular oxidative damage, which can translate into altered cellular functions and as final result, cell death (Halliwell & Gutteridge, 2015). The cerebral tissue is very prone to oxidative imbalance because it is very rich in polyunsaturated fatty acids (PUFAs), has a high metabolic oxidative rate and content of transition metals which together act as potent prooxidants. In addition to brain insufficient antioxidant defines. Depending on the substrate attacked by the free radicals, oxidative stress will manifest as protein, DNA and RNA oxidation or lipid peroxidation (Friedman, 2011).

Increase in oxidation markers and decrease in antioxidant markers in blood, cerebrospinal fluid and in postmortem brain samples of patients with dementia are reported in many studies (Cristalli et al., 2012; Popa-Wagner et al., 2013; Schrag et al., 2013; Chang et al., 2014; Niedzielska et al., 2016). The association between APOE genotype and the cellular stress response in dementia patients yielded inconsistent results. While some studies have shown that APOE4 is positively associated with markers of oxidative stress and negatively associated with antioxidant defense markers compared to APOE3 and APOE2 (Chico et al., 2013; Dose et al., 2016). Non significant differences was reported between the APOE isoforms and specific antioxidative properties by Zito et al. (2013) and López-Riquelme et al. (2016). The overall purpose of this research is to evaluate the possible relationship between APOE genotype and serum level of antioxidant in Saudi patients with dementia of different types.

2 Methods and materials

2.1 Subjects

Eighty elderly Saudi subjects included in this research. They were classified into two groups viz., (i) Control group was recruited from out-patient sections of the Department of Laboratory, Alhada Armed Force Hospital in Taif region (ii) another group was patients with dementia were recruited from the Home Visit Unit, Prince Mansour Military Hospital in Taif region, Saudi Arabia from January to May 2017. The diagnosis of mild, moderate or severe dementia accomplished by a geriatric consultant based on physical examination and neurological tests. Each group has (20 Females, 20 Males) totally 40 subjects with onset age of 65 years or older.

Written consents were obtained from subjects or their caregivers. Unit of Medical Research Committee in Armed Forces Hospitals approved this study. The experimental work of this study was conducted at the Experimental Biochemistry Unit and Central Labs, King Fahd Medical Research Centre (KFMRC), Jeddah, Saudi Arabia.

2.2 Blood Collections

Blood collections were performed according to the standard process. Whole blood samples were drawn from the antecubital vein of patients with dementia and age-matched control. Blood sample were collected in gel serum separation tubes (SST) for antioxidant capacity and lipid peroxidation tests and into (K2EDTA) anticoagulated tubes for DNA extraction. Collected blood samples were kept in a thermal insulated box along with packs of ice through transport. The yellow gel tubes were allowed to clot at room temperature for 30 minutes and centrifuged at 2500 xg for 10 min at 4˚C. Then, serum supernatant was removed and divided into 0.5 ml aliquots and stored at around -80˚C until analysis. The Lavender tubes were used for DNA extraction stored in refrigerator at 4˚C.

2.3 Antioxidant Capacity and Lipid peroxidation Assessment

Ferric Reducing Antioxidant Power (FRAP) as antioxidant capacity indicator and Thiobarbituric Acid Reactive Substances (TBARS) concentrations as one of final product of lipid peroxidation were measured according to kit manufacturer protocols. FRAP Assay Kit (CellbioLab,USA, Cat. no. STA-859) and TBARS Assay Kit (CaymanChem USA, Cat. no. 10009055).

2.4 Measurement of Antioxidant capacity

Ferric Reducing Antioxidant Power (FRAP) assay is redox-dependent colorimetric assay. The principle based on the highly-cited work of Benzie & Strain(1996) which antioxidants present within the sample donated electrons to Ferric iron (Fe3+) which lead to reduced them to the ferrous form (Fe2+). The iron colorimetric probe complex developed a dark blue color produced upon reduction, which can be measured at 540-600 nm (Benzie & Strain, 1996).

2.5 Measurement of Lipid Peroxidation

A well-established method for screening and monitoring lipid peroxidation is the measurement of Thiobarbituric Acid Reactive Substances (TBARS) which are naturally present in biological samples and reported in malonaldehyde (MDA) equivalents, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. In the presence of heat and acid, MDA in the samples was reacted with TBA to produce a colored end product which can be measured calorimetrically at wavelength 530-540 nm.
2.6 DNA Extraction

Genomic DNA was extracted from whole blood samples using Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, USA, Cat. no. K0782). The extracted DNA was stored at -20°C for PCR amplification. Concentration and purity of the extracted DNA was calculated automatically by Nanodrop2000c instrument from Thermo Scientific (USA).

2.7 Polymerase chain reaction

For Polymerase Chain Reaction (PCR), the reactions were prepared using GoTaq®Green PCR Master Mix (Promega, USA, Cat. no. M7122). The primers were developed from Macrogen. The forward primer was (5'-ACA GAA TTC GCC CCG GCC TGG TAC AC-3') and the reverse one was (5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3') as described by Emi et al. (1988). The reaction mix (50µl) contained 2X reaction buffer, 4µM MgCl2, 4µM deoxyribonucleoside triphosphates, 0.2µM of each primer, 0.45 U Taq DNA polymerase, 0.1 µM of Dimethyl Sulfoxide (DMSO) and 10-30 ng of DNA template. The total reaction volume was made up to 50µl with nuclease free water. The amplification conditions consisted of an initial denaturation at 95°C for 1 min, 35 cycles of denaturation at 95 °C for 1 min, an annealing at 65 °C for 1 min and an extension at 72 °C for 1 min, followed by a final extension at 72°C for 5 min and ended at hold at 4°C. To verify PCR product, 2% agarose ethidium bromide stained gel was used.

2.8 Genotyping of Apolipoprotein E

Amplified PCR products were digested with HhaI restriction enzyme which was collected from Thermo Scientific (Cat. No. ER1851). This enzyme was used according to the supplier recommended protocols (Thermo Scientific, USA). By using the thermal cycler, the reaction was incubated in 37°C for 16 hour which is the activation temperature, then the enzyme was inactivated by adding 0.5 M EDTA, pH 8.0. The restriction enzyme results were determined in 8% non-denaturing polyacrylamide ethidium bromide stained gels.

2.9 Statistical analysis

All statistical analyses were performed using computer program package (SPSS, 16.0.). Descriptive data were given as mean ± standard deviation (SD). Differences among groups were tested using the t-test. Differences in the distribution of the alleles and genotypes between the females and males in each groups were examined with the chi-square analysis. Rare genotypes (n=0 in any group) were excluded from the analysis. Mean of data was compared for different genotypes by analysis of variance (ANOVA). The differences in genotype frequencies were considered statistically significant for p value < 0.05.

3 Results

3.1 The Main Characteristics of the Study Group

The subjects in this study were classified according to cognitive function as two groups control (20 Females, 20 Males) and demented patients (20 Females, 20 Males) totally 40 subjects in each group. The biochemical parameters of the two groups are shown in (Table 1). The age between demented patients and control was exhibited a non-significant differences p value= 0.0683>0.05. The antioxidant capacity (FRAP) values between demented patients and control showed a significant difference p value= 0.0125˂0.05, but non-significant between females and males in each group. The lipid peroxidation (MDA) values are

| Parameters               | Non-Demented Mean ± SD | Demented Mean ± SD | P1     | P2    | P3     |
|--------------------------|------------------------|--------------------|--------|-------|--------|
| Age (years)              | 76.67±2.64             | 78.46±5.68         | 78.61± 5.23 | 81.35±6.93 | 0.2826 | 0.1813 | 0.0683 |
| (FRAP) Assay Iron (II) Concentration (µM) | 199.79±58.74 | 189.57±84.75 | 164.81±55.12 | 155.01±35.81 | 0.6601 | 0.5087 | 0.0125* |
| SUM                      | 194.68±72.16           | 159.91±46.15       |        |       |        |
| (TBARS) Assay MDA Concentration(µM) | 7.97±3.80 | 9.02±3.21          | 13.10±6.43 | 9.26±4.95 | 0.3516 | 0.0413* | 0.0167* |
| SUM                      | 8.49±3.51              | 11.18±5.99         |        |       |        |

* Significant difference; P¹ Value for female and male Control group; P² Value for female and male Demented; P³ Value for Control group and Demented; The values represented as the mean ± standard deviation (SD)
significantly different p-value =0.0167<0.05 between demented patients and control, also between females and males in demented group p-value= 0.0413<0.05

3.2 Analysis of the HhaI Polymorphisms in the APOE Gene

The PCR product in samples collected from Saudi subjects, ~ 244-bp has been shown in figure 1. The samples were digested with the HhaI restriction enzyme. The main fragment lengths of digested products for E2 were 91 and 83 bp, E3 were 91, 48 and 35 bp, and for E4 were 72, 48 and 35 bp (Figure 2). For APOE, three alleles and four genotypes (E2/3, E3/3, E3/4 and E4/4) were identified. The allele frequencies were 87.5% for E3, 10.6% for E4 and 1.9% for E2. The most frequent genotype was the E3/3 with 76.2% allele frequency. On the other hand, none of the studied subjects had E2/4 or E2/2 genotype. The distribution of the allele and genotypes of ApoE has been shown in table 2.

3.3 Statistical Analysis of the APOE Alleles and Genotypes Frequencies in Saudi Subject with and without Dementia

Frequencies of APOE alleles and genotypes in the two studied groups with respective to gender were shown in table 3. The distribution of alleles frequency of E3, E4 and E2 differ significantly between demented and control groups (p = 0.0168 <0.05). E3 allele was more frequent in both control and patient groups. The frequency of E2 allele was more frequent in control group compared with demented patients. In contrast, the frequency of E4 allele was more frequent in demented patients compared with control group. Also, it was highly significant between females and males of demented group (p value=0.0423<0.05). The distribution of APOE genotype was significantly different between controls and patients (p-value=0.0447<0.05). Further, E3/E3 genotype occurred more frequently in both controls and patients. On the other hand, the frequency of E3/E4 and E4/E4 were more frequent in demented group compared with controls.

3.4 Characteristics of Subjects According to their APOE Genotype

The study of sample characteristics with respect to APOE genotypes are shown in table 4. In all polymorphisms, there were no significant differences

| APOE gene Polymorphism | Non-Demented n=40 | (100%) | Demented n=40 | (100%) | Total n=80 | (100%) |
|------------------------|--------------------|--------|---------------|--------|------------|--------|
| Allele                 |                    |        |               |        |            |        |
| E3                     | 75                 | 93.7   | 65            | 81.2   | 140        | 87.5   |
| E4                     | 3                  | 3.8    | 14            | 17.6   | 17         | 10.6   |
| E2                     | 2                  | 2.5    | 1             | 1.2    | 3          | 1.9    |
| Total                  | 80                 | 100    | 80            | 100    | 160        | 100    |
| Genotype               |                    |        |               |        |            |        |
| E3/E3                  | 35                 | 87.5   | 26            | 65     | 61         | 76.2   |
| E3/E4                  | 3                  | 7.5    | 12            | 30     | 15         | 18.8   |
| E4/E4                  | 0                  | 0      | 1             | 2.5    | 1          | 1.2    |
| E3/E2                  | 2                  | 5      | 1             | 2.5    | 3          | 3.8    |
| Total                  | 40                 | 100    | 40            | 100    | 80         | 100    |
Assessment of Possible Association between Antioxidant Levels and APOE Gene Polymorphism

Table 4 Characteristics of Subjects with and without Dementia According to their APOE Genotype

| Genotypes     | n= (60) | n= (10) | n= (3) | P-value |
|---------------|---------|---------|--------|---------|
| Age (years)   | 77.2±6.38 | 78.0±5.34 | 82.0±17.58 | 0.4708  |
| Fe²⁺ Concentration (µM) | 178.6±66.38 | 164.1±66.48 | 220.5±50.24 | 0.3447  |
| MDA Concentration (µM) | 9.6±4.98 | 11.5±5.27 | 5.4±2.71 | 0.1156  |

Values are mean ± SD      FRAP value= Antioxidant Activity      MDA value= Lipid Peroxidation

(p-value > 0.05) in age, antioxidant capacity and lipid peroxidation level between different genotype groups.

4 Discussion

Studies on the influence of the APOE genotype on antioxidant activities in dementia patients yielded conflicting results. Apolipoprotein E has been investigated in several experimental settings regarding its role in cellular oxidative stress generation and antioxidant responses in isomorphism dependent manner. In relation to that, it has been reported that patients carrying E4 allele are more susceptible to lipid peroxidation. On the other hand, the E2 and E3 isoforms possess greater antioxidant activity (Pulido et al., 2005; Baldeiras et al., 2008; Chico et al., 2013; Dose et al., 2016). Other studies showed that APOE genotype and oxidative stress or antioxidant activities are independent risk factors for dementia (Ihara et al., 2000; Zito et al., 2013; López-Riquelme et al., 2016).

Findings of present study suggested the involvement of oxidative stress in dementia pathogenesis. The mean value of serum antioxidant capacity was significantly reduced in dementia patients group as compared to the control (p-value= 0.0125 < 0.05) but there was no significant difference between females and males in each group. Similar results are reported by various researchers (Guidi et al., 2006; Negahdar et al., 2015; Moslemnejad et al., 2016). In addition, MDA levels as lipid peroxidation indicator showed a significantly higher concentration on patient compared to control group (p-value= 0.0167 < 0.05). Also, MDA levels were significantly higher in females comparing to male in dementia group (p-value= 0.0413 < 0.05). While in case of control group, non-significant difference was reported between females and males group. This observation was similar from previous publications that reported significant differences not only in MDA levels but also in various oxidative stress biomarkers like protein oxidation and DNA oxidation (Schrag et al., 2013; Chang et al., 2014; Iova et al., 2014; López-Riquelme et al., 2016). Demented females had higher levels of malondialdehyde than males that might be due to their higher prevalence of vascular diseases and lipid disorder.

In general, results of present study consist with the hypothesis that suggest the role of oxidative stress in the physiopathology of dementia as it is involved in the crucial events leading to the neural death (Cervellati et al., 2014; Luca et al., 2015). However, other studies have shown no clear association between oxidative stress and dementia (Pulido et al., 2005; Zafrilla et al., 2006; Sekler et al., 2008; Chang et al., 2014). On the other hand, demented patients were slightly older than control groups that could also responsible for increased oxidative stress and decrease antioxidant markers (Cervellati et al., 2014).

Apolipoprotein E is fundamentally involved in the lipid homeostasis in an isoform-dependent manner. The APOE3 is often associated with normal cholesterol levels. On the other hand, APOE2 and APOE4 isoforms are related to lipid abnormalities and risks of specific diseases (Huang &Mahley, 2014).

The APOE4 was shown to be associated with age-related diseases, including Alzheimer’s disease, therefore an increase in mortality risk with advanced age (Dose et al., 2016). The APOE4 is also associated with an increased risk of mild cognitive impairment, vascular dementia diseases, Lewy body disease and frontotemporal dementia. However, most studies have failed to report associations between APOE4 and susceptibility to Parkinson’s disease and PD-associated dementia (Rubino et al., 2013; Zhou et al., 2014; Wang et al., 2014; Rohn, 2014; Walker et al., 2015; Yan et al., 2016; Chen et al., 2016).

The frequency of the APOE alleles showed that the most common isoform was APOE3 in demented patients and control as 81.2% and 93.7%, respectively. Moreover, the frequency of allele E4 was higher in subjects with dementia as 17.6% compared to 3.8% in control, respectively. Also, it was more frequent in females compared to males in demented group (value of p=0.0423). Finally, the frequency of allele E2 was less common as 2.5% and 1.2%, respectively in control and demented group. Concluded that there was a significant difference between alleles distribution in study groups (value of p=0.0168).

In the same manner, the genotype frequency has significantly different between demented patients and control group (value of p=0.0447). Four genotypes were detected (E3/E3, E3/E4, E4/E4, and E3/E2) with prevalence percentage of (65, 30, 2.5 and 2.5, respectively) in demented patients and (87.5, 7.5, 0 and 5, respectively) in control group. These indicate that APOE4 is associated with dementia and cognitive decline, while APOE2 has protective effects. Our results were similar to those reported in many previous investigations (Farrer et al., 1997;
Borenstein et al., 2010; Chen et al., 2016). In contrast to Saudi study that concluded that APOE4 allele was equally frequent in patients with or without dementia. The differences in the prevalence of E3/E3 and E3/E4 in persons with and without dementia were nonsignificant (p > 0.05). Other genotypes with an E2 allele were absent in the tested population (Al-Khedhairy, 2004).

In our research, subjects were classified by their ApoE genotype into three groups: sixty-one subjects (26 dementia patients and 35 controls) were included in the ApoE 3/3 group, sixteen subjects in the APOE 3/4 or 4/4 group (13 dementia patients and 3 controls) and three subjects in the APOE 3/2 group (1 dementia patient and 2 controls). Then, we examined the association of antioxidant capacity and lipid peroxidation levels with different APOE genotypes.

Then, we examined the association of antioxidant capacity and lipid peroxidation levels with different APOE genotypes. Data analysis showed that there is no significant difference was found between the antioxidant level and lipid peroxidation results according to the differences in ApoE genotypes (p=0.3447 and 0.1156>0.05), respectively. These findings support the hypothesis that demonstrate the presence of E4 allele and decline antioxidant level with elevated lipid peroxidation are independent risk factors for dementia. and they are contribute to the pathogenic cascade in dementia by different pathways (Ihara et al., 2000; Zito et al., 2013; López-Riquelme et al., 2016). In conclusion, our study indicated that the APOE4 allele and oxidative stress are separated risk factor in the pathogenesis of dementia in the Saudi population.

Results of this study recommend that using the same methodology with larger sample size in the same population to determine whether APOE gene and oxidative stress are work together in the pathogenesis of dementia or separately. Moreover, supplementary Antioxidants are recommended to patients with dementia.

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research or publication of this article.

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