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Original Research Article

Antioxidant Capacity is Decreased in Alzheimer's Disease and Mild Cognitive Impairment Patients

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ABSTRACT

We investigated through measurements in serum, the occurrence of oxidative stress in patients with Alzheimer's disease (AD), mild cognitive impairment (MCI) and healthy elderly controls. Possible correlations between a genetic risk factor for AD, the allele ε4 of the apolipoprotein E (APOE) gene, and oxidative stress were also investigated. Through Thiobarbituric Acid Reactive Substances (TBARS) assay serum lipid peroxidation products of AD patients, MCI patients and controls were measured. We also analyzed the participants' serum antioxidant status through 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction. No difference between the groups was observed concerning TBARS levels (p = 0.212). Controls had a higher antioxidant status compared to AD (controls: 0.43 ± 0.061 O.D; AD: 0.39 ± 0.065 O.D), p = 0.002, and MCI patients (0.381 ± 0.065 O.D), p = 0.001. No difference concerning antioxidant status or TBARS levels was associated with the ε4 APOE allele. Oxidative stress in MCI and AD patients seems to be evidenced in serum by a reduction of the antioxidant system capacity rather than change in the TBARS levels. MTT assay for evaluation of antioxidant status could be helpful in therapeutic intervention and guide changes in the life-style of individuals in early stages of cognition impairment.

Keywords: Alzheimer's disease; Biochemical markers; Aging; Dementia; Molecular markers.

INTRODUCTION

Cognitive decline and dementia affect the quality and life span in the elderly. [1]

Free radicals are a natural outcome of energy production, phagocytosis, growth regulation, intercellular signaling and synthesis of important biological components. Disturbances in these processes can increase the amount of oxygen and nitrogen reactive species. [2] The reactive oxygen species (ROS) can modify and change the structure of DNA, lipids and protein (such as complement proteins, signal transducers, embryogenesis regulators, transcription factors). [3] The imbalance between reactive ROS and antioxidants is known as oxidative stress. Evidences point to oxidative stress as a common event of several chronic and inflammatory diseases such as atherosclerosis, arthritis and diabetes. [4] Moreover, this event is thought

to play an important role in Alzheimer's disease (AD). [5]

In Alzheimer's disease, the occurrence of reactions involving ROS leads to a vicious circle of chronic neuro-inflammation that keeps promoting oxidative stress and thus contributes to irreversible dysfunction and cell death. [3] Oxidative stress can lead to an increased production and accumulation of β-amyloid (Aβ) peptide in the brain by enhancing the activity of secretases [6] or modifying proteins in the blood- brain- barrier making it more permeable to Aβ [7] Aβ in turn is highly associated to neurodegeneration and can cause oxidative stress itself. [8]

The ability of ROS to modify lipid membranes is well characterized and increased lipid peroxidation products are found in the brain of AD patients. [3,9] Lipid peroxidation generates several aldehydes and isoprostanes that can be used to assess the oxidative status of biological material. Malondialdehyde (MDA) and F2-isoprostane are the main biomarkers for oxidative stress. [10] MDA can react with thiobarbituric acid and generate a colorimetric product that can be spectrophotometrically read. The product of this reaction corresponds to the thiobarbituric acid reactive substances (TBARS) assay and it has been used for assessing cerebral aging. [11]

Increase in ROS, however, only results in oxidative stress when there is an insufficient antioxidant response. [4] Therefore, the evaluation of the antioxidant capacity of biological materials such as plasma or serum can help the characterization of the oxidative stress. Antioxidants can act enzymatically, as glutathione peroxidase (GPx), catalase, superoxide dismutase (SOD) or non-enzymatically, as uric acid, glutathione, histidine peptides, and proteins bound to iron (transferrin and ferritin). Antioxidants as vitamins and flavonoids as well as poliflavonoids are acquired through diet. [10]

Experimental studies *in vitro* and *in vivo* suggest that the use of antidepressants or acetylcholinesterase inhibitors may decrease ROS generation or increase the activity of antioxidant enzymes. [12,13] Some of these results were replicated in humans, mainly regarding antidepressants, and some still need further elucidation. [14,15] Since medication use may influence oxidative stress it should be considered when assessing it.

A diet of fruits and vegetables containing polyphenolic compounds, known for their high antioxidant activity, has been associated with neuroprotection, better cognitive performance and motor function. [16] This current knowledge points to a possible important role for the antioxidant status in the context of Alzheimer's disease.

In this study we investigated the oxidative stress by measuring the TBARS and -3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction in the serum of AD patients compared to patients with mild cognitive impairment (MCI) and healthy controls individuals. MCI corresponds to a subtle but measureable memory loss which is greater than expected for a specific age range. [17] Correlations between the above-cited parameters and the inflammatory marker C-reactive protein (CRP) were performed because inflammation is described as a considerable event in the context of oxidative stress. Also, we intended to assess the effect of the APOE ε4 polymorphism on the oxidative profile since genotype ε4ε4 was associated to a lower plasma antioxidant capacity. [18]

MATERIALS AND METHODS

Subjects

The present case-control study consisted of 53 healthy controls, 35 patients with mild cognitive impairment (MCI) and 48 with Alzheimer's disease. The participants' ages varied from 61 to 89 years old and the three groups were age-homogeneous (Table 1). None of the participants were in use of anti-inflammatory drugs. Screening tests were used for accessing the mental state of the

participants and diagnosing AD or MCI: Mini-Mental State Examination (MMSE) with cut-off limits adjusted according to the education level, CERAD Protocol, Activities of Daily Living Scale, Instrumental activities of daily living and Hachinski Ischemic Scale (< 2).

The diagnostic criteria for amnestic MCI was conducted according to Albert *et al*. [19] The diagnosis of probable AD was based on the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), and criteria developed by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). Demographic data and clinical characteristics of the participants were obtained through interview at the moment of the blood collection or through review of medical records. A thorough geriatric assessment was performed in the eligible patients which medical history, physical and laboratorial screening tests, brain imaging exams and neuropsychological tests, when necessary.

The individuals enrolled in the control group presented no personal or familial (first degree) history of neuropsychiatric diseases. They were submitted to clinical tests to exclude other psychiatric disorders. Other diseases were not observed in this group, however, some individuals used antilipidemic, antihypertensive and / or hypoglycemic drugs.

The study was approved by the Ethics Committee of Federal University of Minas Gerais, Brazil, and an informed consent was signed by all the participants after the research was explained to them. Consent was only given by participants who presented a MMSE score compatible to a non-demented state. Otherwise consent was obtained from relatives or caregivers.

Samples of 5mL of whole blood were collected from each participant in tubes with EDTA and centrifuged. Afterward, serum samples were aliquoted and frozen at -80 ˚C until the analysis was performed. Highly sensitive Near Infrared Particle Immunoassay rate methodology (IMMAGE® Immunochemistry systems, Beckman Coulter - Galway, Ireland) was used to measure C Reactive Protein serum levels and rule out patients who had an ongoing acute inflammation process. All the elected participants had levels under 10 mg/L and their results regarding CRP measurement were used in correlation analysis with TBARS and MTT.

TBARS assay

Lipid peroxidation was evaluated through a "in house" TBARS assay, in which MDA forms an adduct with thiobarbituric acid. We followed the protocol described by Draper & Hadley [20] with modifications. Briefly, 100 µL of the solution: TBA 1%, NaOH 0,05 M and BHT 0,1 mM, as well as 50 µL of phosphoric acid 14% were added to 100 µL of serum in eppendorf tubes. The samples were incubated in dry bath for 25 minutes at 98°C, and then frozen for 10 minutes. Afterward, 375 µL of butanol were added to the tubes which were then vortexed for 10 seconds and centrifuged at 2000 RPM for 5 minutes. Impurities in the supernatant were spectrophotometrically read in 600 nm and their values were discounted from the absorbance obtained at 532 nm.

MTT assay

The serum antioxidant status was measured trough the MTT die reduction, in which circulating antioxidant elements such as ascorbate, urate, α-tocopherol (vitamin E), albumin and sulfhydryl proteins reduce the MTT salt, generating a formazan colorimetric final product, read at 570 nm by spectrophotometer. The protocol described by Reis *et al*. [21] was applied to serum and the result was given as units of optical density (O.D).

Genomic DNA for *APOE* genotyping was amplified by polymerase chain reaction (PCR), followed by digestion with *HhaI* and restriction fragment length polymorphism (RFLP) analysis, as previously described by Hixson & Vernier. [22]

Statistical analysis

Normality was tested for TBARS and MTT values by the Shapiro-Wilk test and they were normally distributed. Comparisons among the experimental were done by One Way ANOVA test followed by LSD post-hoc test. Comparisons between ε4 carriers and non-carriers were performed by T-test. Spearman's test was performed to examine the correlation between MTT or TBARS and CRP since the last one has shown a non-normal distribution. All statistical tests were two-tailed and performed using a significance level of $p < 0.05$. Statistical analyses were performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

As shown in Table 1, there were no differences among the three groups regarding statin use and diabetes frequency. We observed that 54.2% of the AD patients were in use of antidepressants and 71.8% were in use of cholinesterase inhibitors (ChEI). The higher frequency of antidepressant use in AD patients compared to the other groups was also expected since such medication is often applied in AD treatment.

The presence of the allele ε4 of the APOE gene, already presented in our previous study, was also analyzed in each group due to its strong association with the incidence of Alzheimer's disease. The allele was more frequent in the MCI and AD groups compared to controls.

Table 1 - Characteristics of the participants in the three groups – AD, MCI and controls.

	Controls (n= 53)	MCI (n= 35)	AD (n= 48)	*p* – value
Age (SD)	73.9 (5.5)	73.2 (7.2)	76.2 (5.5)	0.053
Gender (W/M)	41/12	19/16	32/16	0.076
APOE ε4	24.5%	62.9 %	54.2%	0.001†,§
Diabetes	18.9%	17.1%	20.8%	0.913
Statins	33.9%	20.0 %	25.0%	0.257
Antidepressants	15.1%	17.1%	54.2%	< 0.001†,‡
ChEI	-	-	71.8%	-

Significant p- value < 0.05. † – significant for control x AD; ‡ - significant for MCI x AD; §-significant for control x MCI. MCI = Mild Cognitive Impairment; AD = Alzheimer's disease; SD = standard deviation; W = women; M = men; APOE ε4 = carrier of ε4 apolipoprotein E allele; ChEI = cholinesterase inhibitors.

No differences were observed among the three studied groups regarding the TBARS serum levels (Controls: 9.24 ± 1.04 μM/L; MCI: 9.28 ± 0.88 μM/L; AD: 9.60 ± 1.21 μM/L; p= 0.212). The MTT assay showed a higher antioxidant status for controls (0.43 ± 0.061 O.D) when compared to the MCI group (0.38 ± 0.065 O.D), p = 0.001; and also compared to AD patients (0.39 ± 0.065 O.D), p = 0.002. There was no significant difference between the MTT results for the MCI and the AD group (p= 0.659) (Fig.1).

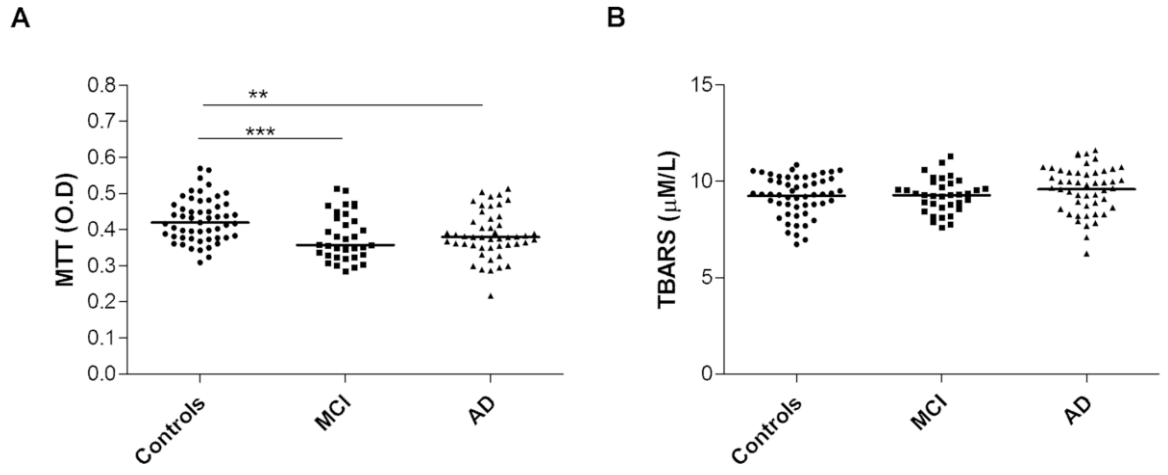

Fig. 1. MTT reduction (a) and TBARS levels (b) in the serum of controls, MCI and AD patients. The middle line represents the mean. ** $p <$ 0.01 and *** p = 0.001. Comparisons were performed through ANOVA followed by LSD pos-hoc test. MCI- Mild Cognitive Impairment; AD- Alzheimer's Disease; O.D – Optical Density.

As described in Table 2, no difference concerning the antioxidant status or the TBARS serum levels was observed when comparing APOE ε4 carriers and non-carriers.

No difference was observed regarding the MTT values for the serum of participants in use of statins compared to the ones who were not in use of statins. Concerning TBARS, statins also did not influence on its serum levels. Regarding antidepressants, no difference was observed in the antioxidant status or the TBARS levels of the participants who used and those who did not use such medication (table 2).

AD patients who were in use of ChEI did not show a significantly different antioxidant status or TBARS levels in comparison with the ones who were not treated with ChEI (table 2).

Table 2 - Antioxidant status (MTT) and TBARS levels according to APOE ε4 presence and use of antidepressant and acetylcholinesterase inhibitors (the last parameter was only considered in AD patients).

	MTT reduction (SD) (O.D)	*p* - value	TBARS (SD) (μM/L)	*p* - value
ε4 carriers	0.39 (0.06)	0.083	9.41 (1.02)	0.928
non-carriers	0.41 (0.07)		9.38 (1.14)	
Statin use	0.41 (0.06)	0.335	9.48 (0.98)	0.490
No statin use	0.40 (0.07)		9.34 (1.12)	
ADS use	0.39 (0.06)	0.464	9.67 (1.17)	0.055
No ADS use	0.40 (0.07)		9.26 (1.03)	
ChEI use	0.38 (0.07)	0.884	9.75 (1.19)	0.552
No ChEI use	0.39 (0.07)		9.60 (1.46)	

Significant p - value < 0.05. MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBARS = Thiobarbituric Acid Reactive Substances; ε4 = apolipoprotein E4 allele; ADS = antidepressants; ChEI = Cholinesterase inhibitors; SD = standard deviation; O.D = optical density.

DISCUSSION

In this study we showed that although no differences were observed among the groups regarding TBARS serum levels, the MTT assay revealed that MCI and AD patients had a lower antioxidant status than age matched healthy controls.

The MTT tetrazolium salt has been long used for evaluation of cell viability and cytotoxicity in tests which are based on the metabolic reduction of MTT into a formazan type product. [23] Medina *et al.* [4] made use of the capacity of circulating antioxidants to reduce MTT and described an assay which is able to inform the antioxidant status of plasma. In the present study the MTT assay applied to serum, showed that MCI and AD patients presented a lower antioxidant status compared to controls.

Sinclair *et al.* [24] had already observed that patients with Alzheimer's disease presented lower levels of vitamin E in their plasma. According to the authors, a degree of imbalance in the antioxidant status is observed in Alzheimer's disease patients or subjects with dementia attributed to vascular disease, pointing out the potential use of antioxidants as therapeutic options. In effect, experiments in animal models of AD have demonstrated that antioxidant substances can improve learning and memory when red grape juice, rich in polyphenol, was given to Alzheimer's rats. [25] In addition, it has been shown that vitamin E reduced Aβ levels in mice if given before AD was actually installed. [26] An AD cohort follow-up of 15 years reported that patients whose diets included vitamin E survived longer than those taking no drugs or ChEI alone. [27]

Foy *et al.* [28] reported that vitamin A, C and E were depleted in AD compared to controls. However, the total antioxidant capacity assay, which measures the inhibition by antioxidants on the formation of peroxyl free radicals, showed no differences between the groups.

In accordance with our results, Rinaldi *et al.* [29] observed that peripheral levels and activities of antioxidants were similarly lower in MCI and AD patients

when compared to controls. MCI and AD patients showed lower levels of vitamin C, vitamin A and vitamin E as well as lower levels of uric acid, lutein, zeaxanthin and α-carotene compared to controls. Also, in keeping with our results Rinaldi *et al*. [29] reported no influence of APOE genotype on the peripheral antioxidant capacity of the participants. In contrast, Pulido *et al*. [18] showed that APOE ε4ε4 subjects had lower plasma antioxidant capacity determined by Ferric-Reducing Ability of Plasma (FRAP) assay. Possibly, this discrepancy is centered in the fact that in our sample only rare patients had E4 allele in homozygosis, whose characteristic, in fact, should contribute to the reduction of the antioxidant state.

No differences regarding TBARS levels were observed among healthy controls, MCI and AD patients in our study. When evaluated in *post-mortem* brain tissue samples, by Ansari & Scheff, [30] TBARS levels showed a disease-dependent increase. TBARS were elevated also in erythrocytes of AD patients. [11] In circulation, however, the results are more controversial. Whilst different research groups have found elevated TBARS in serum/plasma of AD patients, [31,32] others, similarly to our study, did not show differences between the two groups. [33,34] Although in the study of Baldeiras *et al*. [34] TBARS plasma levels were not different between controls and AD patients, there was an increased lipid peroxidation in red cells of MCI and AD patients who were APOE ε4 carriers. In agreement with our findings, although specifically MDA instead of TBARS, have been evaluated, Fernandes *et al*. [35] did not find a correlation between APOE genotype and MDA plasma levels of controls and AD patients. The cited controversies might be related to differences among the studies regarding patient selection, age-range of the participants, methodology and assessment of diet, smoking and supplement use.

Although it was reported that certain antidepressants and acetylcholinesterase inhibitors may be able to influence ROS generation or antioxidant capacity, [12,13] in accordance with other findings [15,36] we did not observe an impact of these variables on the TBARS or MTT measurement in our study. Other variables not controlled in the present study such as use of vitamin supplements may be contributing to counteract the effect of these drugs. Other factors such as the type of drug, dosage and time of use with potential impact on the evaluation of oxidative stress cannot be ruled out either.

Despite being practical and applicable to serum or plasma, the TBARS assay has limitations due to reactions involving thiobarbituric acid and non-lipid moieties. [33] In addition, as MDA can be absorbed through diet and excreted in urine, the amount of lipid peroxidation can be altered by diet and chronic renal insufficiency. Moreover, comorbidities such as diabetes and cardiovascular diseases can increase the TBARS levels. [37] It should be noted that, like the great majority of studies, one limitation of the present study was the impossibility of controlling the participants' diet. However, none of them had renal insufficiency attested by normal levels of creatinine.

In the present study, diabetes was homogeneously present in the three groups studied. However, the limitations cited in relation to the test reaction and the need for a more rigorous selection of participants regarding diet and specific clinical conditions may have influenced the results of this study. Individual habits regarding exercise can also interfere with ROS generation. [38,39] However, due to the fact that patients with AD presented greater difficulty in practicing physical activity, this variable was not evaluated.

The assessment of the antioxidant capacity by the MTT assay is comparable with the lipid peroxidation inhibitory assay and can represent important gains as it is inexpensive, fast and simple methodology. [40] To our knowledge, our study is one of the first records of comparative antioxidant evaluation between Alzheimer's and MCI

patients through the MTT test. Since abnormal oxidative stress is an established characteristic of Alzheimer's disease and as our results have already indicated a reduced antioxidant capacity of plasma in the MCI stage, the importance of investigating oxidative status in elderly individuals who are not yet demented is reinforced. The finding of an abnormal oxidative stress would strongly suggest the need to adopt therapeutic measures aimed at postponing the onset of cognitive decline. On the other hand, the practicality, speed and low cost of the methods used in the present study should encourage the design of clinical studies aiming to evaluate cognitive changes after antioxidant interventions. With more studies confirming our results, the decrease in the antioxidant capacity could be easily determined and may constitute an additional tool for characterizing cognitive decline. Such characterization could lead to more efficient interventions, including a possible use of antioxidant supplements.

Our results are partially in agreement with the meta-analysis performed by Schrag *et al*. [41] who reported a significant oxidative damage in peripheral blood early in the process of neurodegeneration. These authors reported increased levels of markers of lipid peroxidation in the blood of patients with Alzheimer's disease and mild cognitive impairment, dysregulation in copper metabolism, and decreased antioxidant capacity. However, none of the major antioxidant enzymes were significantly diminished while a significant decrease of non-enzymatic antioxidants in the blood (particularly uric acid, vitamins A, E and C, α and β-carotene) was observed by the same authors.

CONCLUSION

Overall, a disruption in the redox balance seems to take place in MCI and AD patients mainly due to a decreased antioxidant capacity. A simple test as the MTT assay that is able to detect such disruption in MCI cases (compared to controls) is of great value, encouraging changes in life-style and diet that might diminish the chances of developing a risk factor for AD.

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Conflict Of Interest

No conflicts of interest exist for any of the authors of this study.

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