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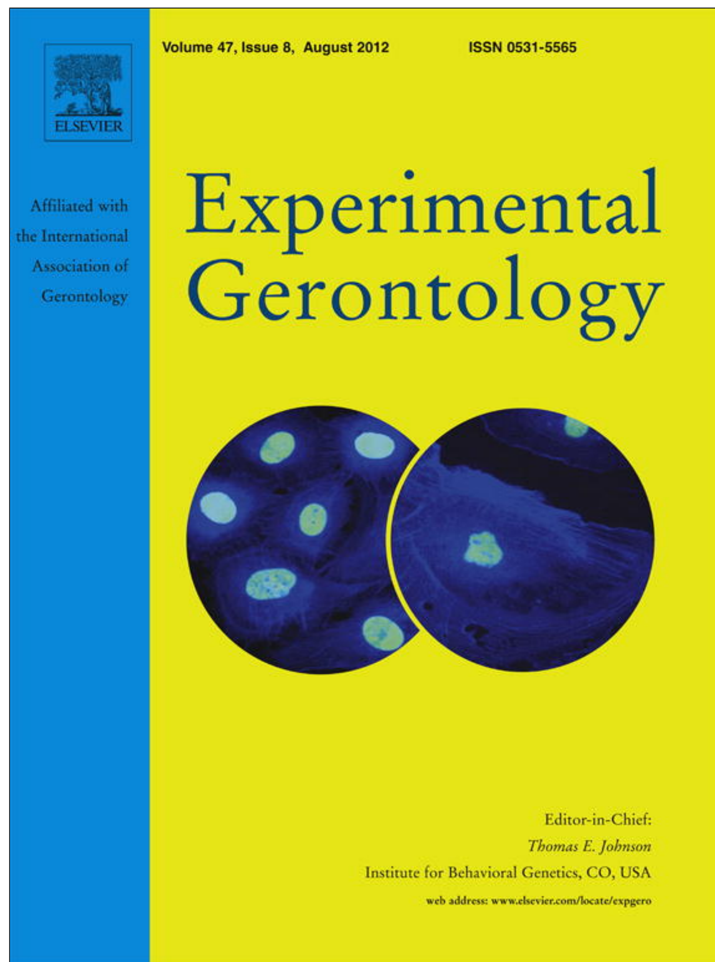

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Plasma oxidative stress parameters in men and women with early stage Alzheimer type dementia

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ABSTRACT

It is well known that oxidative stress is one of the earliest events in Alzheimer's disease pathogenesis, indicating that may play a key role in this disease. In our study, we measured the levels of oxidative stress indicators (TBARS and protein carbonyls content) and the non-enzymatic (glutathione (GSH) and oxidized glutathione (GSSG)) and enzymatic (glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD)) defense systems in the plasma of 46 patients diagnosed of ATD and 46 age-matched controls. We found decreased levels in total GSH in ATD patients, although healthy control women showed lower levels of total GSH than healthy control men. On the contrary, we found increased levels of TBARS and carbonyl groups content in ATD patients in both genders. The activity of the plasma antioxidant enzymes showed no changes for SOD activity in ATD patients, independently of the gender, although western blot analysis showed an increase in SOD-1 protein. CAT activity was also decreased in ATD patients, although this decrease is mainly due to the decrease found in men but not in women. However, western blot analysis did not show differences in CAT protein between controls and ATD patients. Finally, a decrease of GPx activity was found in ATD patients in both genders. However, as with CAT protein, western blot analysis did not show differences in GPx protein between controls and ATD patients. Our results suggest that there is a defect in the antioxidant defense system that is incapable of responding to increased free radical production, which may lead to oxidative damage and the development of the pathological alterations that characterize the neurodegenerative disorder of patients with ATD. Thus, oxidative damage could be one important aspect for the onset of ATD and oxidative stress markers could be useful to diagnose the illness in their earliest stages through both non-invasive, reliable and cost-affordable methods.
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1. Introduction

All the aerobic cells generate reactive oxidative species, particularly in the oxidation–reduction reactions necessary for the generation of ATP. In specialized cells with high metabolic activity, such as neurons, the number of free radicals produced is estimated about 10^{11} reactive oxidative species/cell/day (Bonda et al., 2010; Petersen et al., 2007). The brain is particularly vulnerable to oxidative stress as a result of the relatively low levels of antioxidants, high levels of polyunsaturated fatty acids and increased need of oxygen (Sultana et al., 2008). There are many evidences that suggest that oxidative stress is one of the earliest events in Alzheimer type dementia (ATD) pathogenesis and plays a key role in the development of the ATD pathology (Bonda et al., 2010; Zhu et al., 2001, 2004, 2007). An accumulation of products of free radical damage in central nervous system (CNS) in subjects with ATD has been described (Butterfield et al., 2006, 2007; Mangialasche et al., 2009) and it is believe that oxidative damage to critical molecules occurs early in the pathogenesis of ATD; perhaps is the earliest feature of an ATD brain (Nunomura et al., 2001; Zhu et al., 2001, 2004, 2007) and precedes pronounced neuropathological alterations (Baldeiras et al., 2008; Lovell and Markesbery, 2007; Nunomura et al., 2001). In fact, some evidences have suggested that the ß-amyloid deposition in ATD neurons may be considered as an effort to protect these cells against damage due to oxidative stress (Bonda et al., 2010; Hayashi et al., 2007; Nakamura et al., 2007).

Enzymatic and non-enzymatic defense systems are responsible to maintain an adequate oxidative state. Thus, glutathione (GSH) plays a significant role as a redox regulator and is required as an essential antioxidant for cellular protection. In the same way, antioxidant enzymes such as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD), also participate in maintain redox state through different pathways. Therefore, the objectives of the current study was to determine in plasma the levels of total glutathione (reduced GSH plus oxidized glutathione (GSSG), thiobarbituric acid reactive substances (TBARS) as index of lipid peroxidation, protein carbonyl content as index of protein oxidation, and the activities and content of the

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antioxidant enzymes (SOD, GPx, and CAT) in early stage ATD patients to clarify its value as biomarkers to diagnose the illness in their earliest stages through both non-invasive, reliable and cost-affordable methods.

2. Material and methods

2.1. Subjects

The subjects of this study were 46 individuals with ATD (20 males; age 76.0 years ± 1.64; 26 females; age 73.96 years ± 1.49) and 46 healthy age-matched controls (16 males; age 73.25 years ± 1.56; 30 females; age 73.83 years ± 1.24). Patients were recruited from the Unit of Neurology of the University Hospital "Ciudad de Jaén" and healthy voluntaries from routine controls. People who take antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs), steroids, vitamins or antioxidant supplements were excluded. Subjects with history of smoking and alcohol intake were also excluded from the study. Subjects received diagnosis of ATD if they met DSM-IV clinical criteria for dementia, and received a diagnosis of probable or possible ATD according to NINCDS/ADRDA (National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association) criteria. People with ATD who had comorbidity with other clinical major neurological illness were excluded.

Cognitive and functional status of ATD patients were assayed by Mini-Mental State Examination (men 23.7 ± 0.92; women 20.7 ± 0.66); Blessed Scale (men 6.50 ± 0.74; women 7.28 ± 0.69) and Brief Cognitive Rating Scale (BCRS) adapted as Functional Assessment Stage (FAST) (men 3.71 ± 0.22; women 3.78 ± 0.16).

Study participants' fasting blood samples were collected in the morning and centrifuged immediately. Plasma samples were stored at −80 °C until measurement.

The research protocol was approved by the local Clinical Research Ethical Committee at University Hospital of Jaén. All patients and their legal's guardians provided written informed consent.

2.2. Oxidative stress parameters assays

2.2.1. Lipid peroxidation assay

Lipid peroxidation was measured by analyzing the amount of thiobarbituric acid reactive substances (TBARS) (Mayas et al., 2002). Twenty five microlitres of each sample was mixed with 100 μL of ice-cold 20% trichloroacetic acid (TCA). After centrifugation, a volume of supernatant was added to an equal volume of 0.67% 4,6-dihydroxypyrimidine-2-thiol (TBA) and the mixture was kept in a boiling water bath for 15 min. Samples were cooled to room temperature and the absorbance at 532 nm was recorded after subtracting blanks containing TCA and TBA in an equal volume. The signal was read against a malondialdehyde (MDA) standard curve and the results were expressed as mg/mL.

2.2.2. Protein oxidation assay

Carbonyl groups content of proteins were determined as previously described (Mayas et al., 2002). Twenty five microlitres of sample was mixed with 100 μL of ice-cold 20% TCA and centrifuged. Protein precipitates were left to react with 2,4-dinitrophenylhydrazine 10 mM for an hour at room temperature in the dark. After the reaction, proteins were precipitated with 20% TCA and unreacted dye was washed twice with 10% TCA. The pellets were dissolved in 1 M NaOH and absorbances were recorded at 360 nm. The results were expressed as nmol per mg of protein using a extinction coefficient of 22.000 cm^{-1} M^{-1}.

2.2.3. Determination of glutathione (GSH) and glutathione disulfide (GSSG)

GSH levels were measured as described by Griffith (1980) with minor modifications. For this purpose, plasma samples were treated with two volumes of a buffer constituted discarded by centrifugation at 10,000 *g* for 5 min at 4 °C. Supernatants containing total GSH were mixed with 0.6 mg/ml of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.248 mg/ml nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was started on addition of 1 U/mL GSSG reductase. Absorbance was measured at 405 nm at 30 °C in a TECAN GENios Plus spectrophotometer every 20 s for 2 min. For GSSG determination, 2-vinylpyridine was used to derivatize GSH. Data are presented as nmol of total GSH (GSH plus GSSG) per mg of total protein.

2.2.4. Catalase activity assay

Plasma samples were processed and analyzed for catalase (E.C.: 1.11.1.6) activity as described by Aebi (1984) with slight modifications by Cohen et al. (1996). Briefly, 10 μL of protein solution was added to 10 mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7.0) and incubated at 30 °C for 1 min. Initial reaction rate was measured from the decrease in absorbance at 240 nm.

2.2.5. Superoxide dismutase assay

SOD activity was as measured according to Paoletti et al. (1986). Ten microlitres of protein solution was mixed with reaction buffer contained 100 mM, TDB buffer (triethanolamide-diethanolamide, pH 7.4), 7.5 mM NADH and relation 1:2 EDTA/MnCl2. To start the reaction, 25 μL of 10 mM mercatoethanol was added. The absorbance at 340 nm between 2 and 15 min was recorded.

2.2.6. Glutathione peroxidase activity assay

GPx activity was measured according to Ellerby and Bredesen (2000). The reaction mixture was formed by 50 mM potassium phosphate (pH 7.4) 25 mM NADPH, 1 mmol/L of GSH, 100 U/ml of yeast GRd. Ten microlitres of protein solution per sample were added and mixed with the reaction mixture in a 96-well dish. The hydroperoxide-independent NADPH consumption rate was recorded for 3 min at 37 °C at 340 nm in an automatic microplate reader (TECAN GENios Plus). Then, 2.5 μL of tert-butyl hydroperoxide was added to start the reaction, mixed, and the overall rate at 340 nm was recorded. The same procedure was carried out in the same reaction volume without the sample protein. This allows subtracting from the total rate, the nonenzymatic rate of GSH oxidation.

2.3. Western blotting

Samples were treated with RIPA buffer. Fifty micrograms of total proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham GE-Healthcare, Buckinghamshire, UK). Protein concentration was determined according to Bradford's method. Proteins were loaded per lane onto a 15% SDS-PAGE gel for Cu-Zn superoxide dismutase (23 kDa), or a 10% SDS-PAGE gel for glutathione peroxidase (92 kDa), and 7.5% SDS-PAGE gel for catalase (64 kDa). After blocking with 5% skim milk in TBS, the membrane was incubated with goat anti-SOD-1 (1:200, Santa Cruz Biotechnology, Inc.), goat anti-GPx-1 (1:200, Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-catalase antibodies (1:200, Santa Cruz Biotechnology, Inc.) overnight at 4 °C. Blots probed with tubulin (1:10,000; Santa Cruz Biotechnology, Inc.) were used as loading controls. The membranes were washed three times with TBS-T. Immunoreactive polypeptide was visualized using horseradish peroxidase conjugated secondary antibodies (anti-goat IgG peroxidase conjugated 1:2000, 1:5000 Santa Cruz Biotechnology, Inc. and anti-mouse IgG peroxidase conjugated 1:2000, Amersham Bioscience) and enhanced-chemiluminescence detection reagents (Amersham Bioscience) following manufacturer-supplied protocols. Inmunoblots were analyzed by ImageLab™ Software version 2.0.1 (Bio-Rad) to provide quantitative values for relative expression of each protein (all normalized to its own loading control). The optical densities of the bands were measured by ImageLab.

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2.4. Statistical analysis

All values represent the mean of the individual determination ± standard error of the mean (SEM). Data were analyzed by ANOVA plus Newman–Keul's test, using IBM Pass V.19 software. Values of $p<0.05$ were considered significant.

3. Results

Significant increased levels of TBARS ($p<0.01$; Fig. 1A) and carbonyl groups content ($p<0.05$; Fig. 2A) were found in plasma of ATD patients, these increases also being observed in men and women ATD patients ($p<0.01$ and $p<0.05$ for TBARS and carbonyl groups content respectively; Figs. 1B and 2B).

On the contrary, we found significant decreased plasma levels in total GSH ($p<0.001$) in ATD patients (Fig. 3A). These decreases were also observed in men and women ATD patients ($p<0.001$) (Fig. 3B). However, healthy control women showed lower levels of total GSH than healthy control men ($p<0.001$).

The activity of the plasma antioxidant enzymes showed no changes for SOD activity in ATD patients, independently of the gender (Fig. 4A and B), although western blot analysis showed a significant increase in SOD-1 protein ($p<0.01$; Fig. 4C). CAT activity was also significantly decreased ($p<0.05$) in ATD patients, although this decrease is mainly due to the significant decrease ($p<0.05$) found in men but not in women (Fig. 5A and B). However, western blot analysis did not show differences in CAT protein between controls and ATD patients (Fig. 5C).

Finally, a significant decrease of GPx activity was found in ATD patients ($p<0.01$) (Fig. 6A.). These decreases were also observed in men ($p<0.01$) and in women ($p<0.01$) with ATD (Fig. 6B). However, as with CAT protein, western blot analysis did not show differences in GPx protein between controls and ATD patients (Fig. 6C).

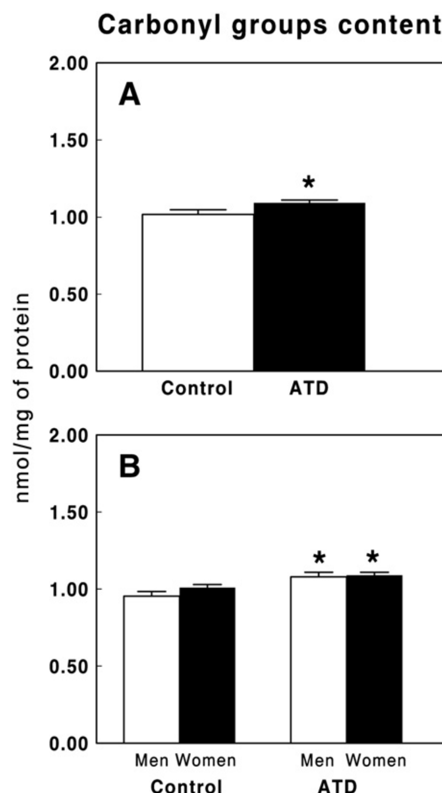

Fig. 2. Carbonyl groups content in plasma of patients with early stage Alzheimer type dementia (ATD) and their respective controls (A) and their gender differences (B). Results are expressed in nmol/mg of protein (Mean ± SEM; $n=46$ (16 control males, 30 control females, 20 ATD males and 26 ATD females); $^{*}p<0.05$).

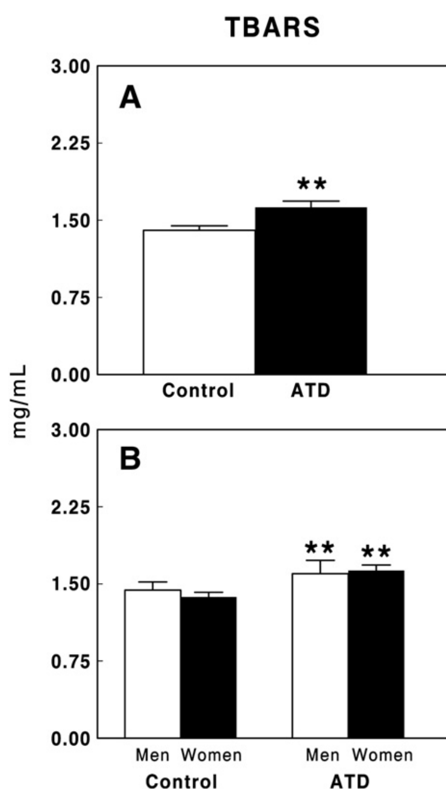

Fig. 1. Thiobarbituric acid reactive substances (TBARS) content in plasma of patients with early stage Alzheimer type dementia (ATD) and their respective controls (A) and their gender differences (B). Results are expressed in mg/mL (Mean ± SEM; $n=46$ (16 control males, 30 control females, 20 ATD males and 26 ATD females); $^{**}p<0.01$).

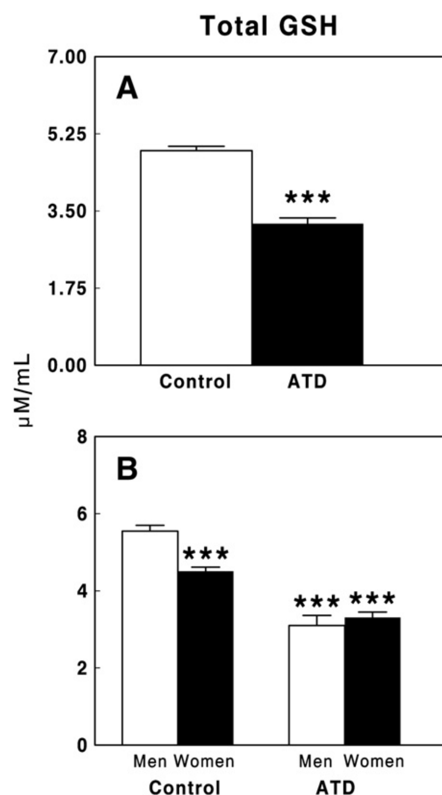

Fig. 3. Total glutathione (GSH) content in plasma of patients with early stage Alzheimer type dementia (ATD) and their respective controls (A) and their gender differences (B). Results are expressed in μM/mL (Mean ± SEM; n = 46 (16 control males, 30 control females, 20 ATD males and 26 ATD females); $^{***}p<0.001$).

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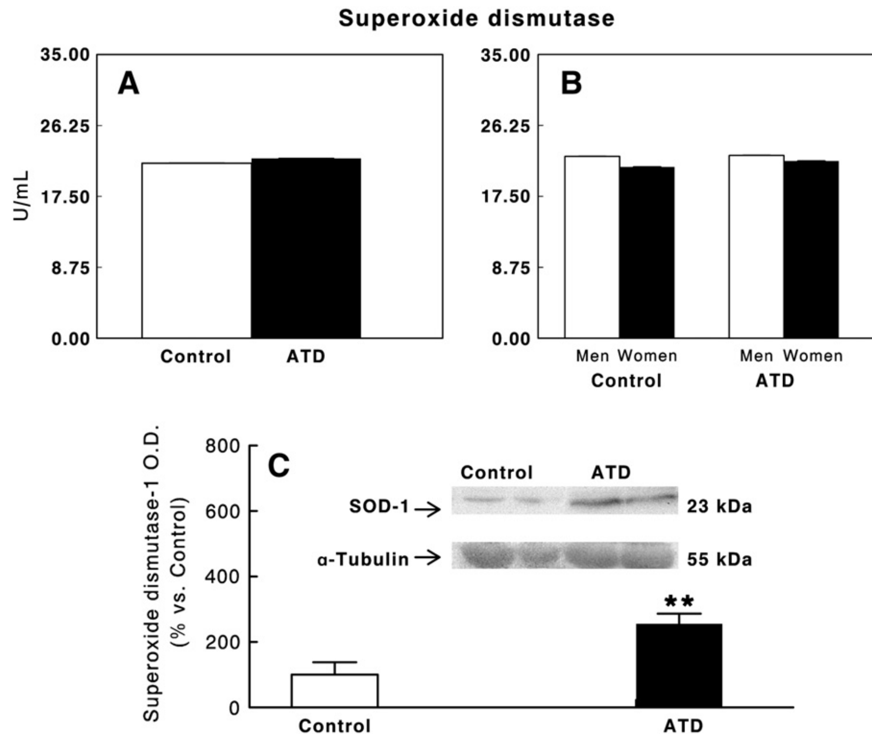

Fig. 4. Cu-Zn Superoxide dismutase (SOD) activity in plasma of patients with early stage Alzheimer type dementia (ATD) and their respective controls (A) and their gender differences (B). Results are expressed in U/mL (Mean ± SEM; n = 46 (16 control males, 30 control females, 20 ATD males and 26 ATD females)). Figure (C) shows a representative western blot showing increased Cu-Zn SOD (23 kDa) plasma protein levels in ATD patients (**$p<0.01$).

4. Discussion

The diagnosis of ATD, as in several other neurodegenerative disorders, is based mainly on clinical symptoms. Thus, research into possible new biomarkers for disease prevention, early detection or disease evolution is of major importance. Furthermore, there is experimental evidence that oxidative damage is the earliest event in cognitive decline. In the present report, we present data that evidence signs of oxidative stress in men and women with earliest ATD. Those signs are, firstly, the significant increase of TBARS and protein carbonyls without

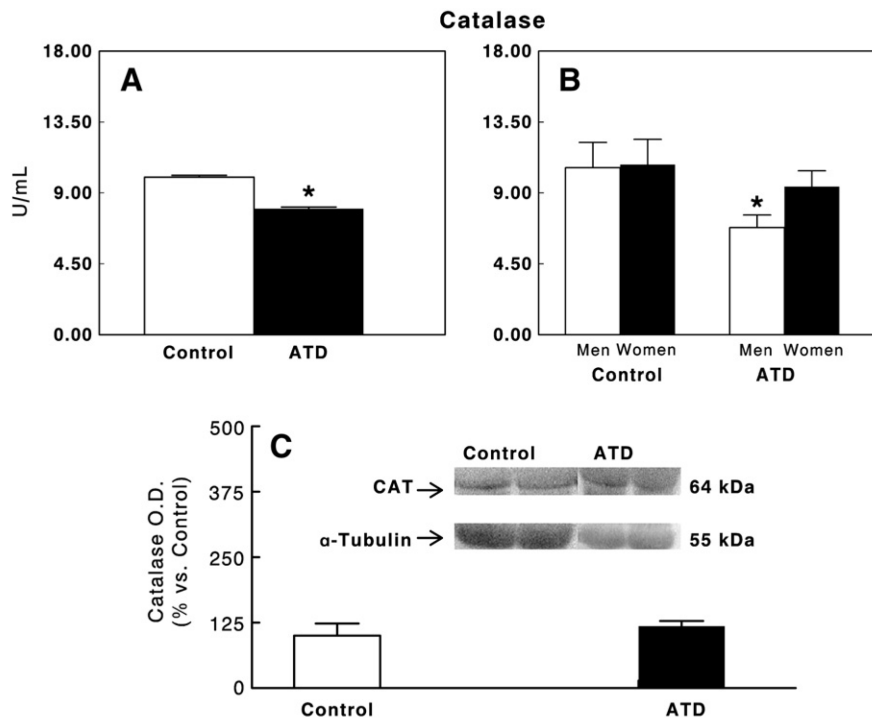

Fig. 5. Catalase (CAT) activity in plasma of patients with early stage Alzheimer type dementia (ATD) and their respective controls (A) and their gender differences (B). Results are expressed in U/mL (Mean ± SEM; n = 46 (16 control males, 30 control females, 20 ATD males and 26 ATD females); *$p<0.05$). Figure (C) shows a representative western blot showing no changes in CAT (64 kDa) plasma protein levels between groups.

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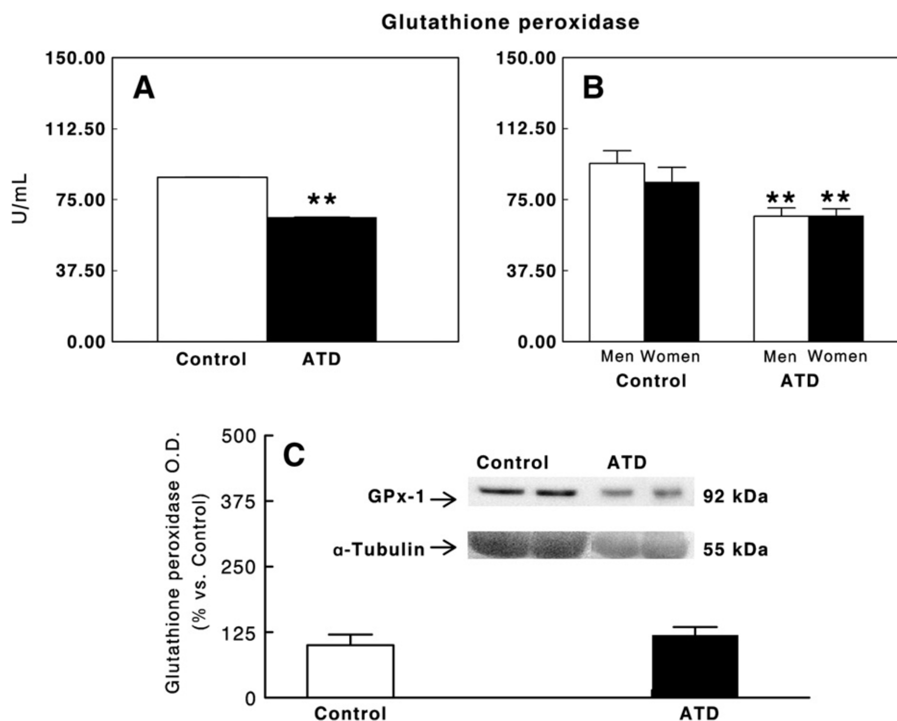

Fig. 6. Glutathione peroxidase (GPx) activity in plasma of patients with early stage Alzheimer type dementia (ATD) and their respective controls (A) and their gender differences (B). Results are expressed in U/mL (Mean ± SEM; n = 46 (16 control males, 30 control females, 20 ATD males and 26 ATD females); $^{**}p<0.01$). Figure (C) shows a representative western blot showing no changes in GPx-1 (92 kDa) plasma protein levels between groups.

differences between genders. TBARS are indicators of lipid peroxidation, whereas protein carbonyls are indicators of protein oxidation. Our data agree with several authors who have previously described these increases (Cristalli et al., 2012; Padurariu et al., 2010). In fact, actually most of the studies described increased levels of TBARS and protein carbonyls in ATD patients. In general, the increase of oxidative processes (lipid and protein oxidation) will lead to the fall of the enzymatic and non-enzymatic antioxidant defense system. In this regard, we have also found the significant decrease of total GSH in plasma of ATD patients in comparison with the healthy controls. GSH is the most important intracellular free thiol and plays an important role as antioxidant defense (Liu et al., 2005). Different authors have reported changes in GSH content in different brain areas with contradictory results (Adams et al., 1991; Gu et al., 1998) and also in blood cells and plasma. In fact, Bermejo et al. (2008) described lower levels of GSH in ATD patients and Liu et al. (2005) analyzing GSH content in plasma did not find changes in ATD patients, but described that GSH content was decreased in the red blood cells from male but not in female ATD patients. In this way we also analyzed gender differences, but our results did not demonstrate gender related differences because we observed lower levels of GSH in men and women with ATD than in healthy men and women. However, our results also agree with those recently reported by Cristalli et al. (2012) who show in ATD patients a decrease in total GSH content in plasma but also in total blood and in erythrocytes, without detecting gender differences. Furthermore, total GSH content decreased progressively along clinical evolution of the patients, also supporting the importance of GSH as a biochemical biomarker for early diagnosis.

Secondly, although we have found no significant differences in the rate of SOD activity in plasma, ATD patients revealed a significant decreased rate of CAT and GPx activities. Under physiological conditions, there may be a balance between the rate of H_2O_2 production via dismutation of superoxide by SOD and its removal by CAT and GPx. Our results may indicate that the first level of the protective cascade of oxidative stress did not change in ATD, but the second and third levels are clearly affected. However, western blot analysis showed an increase on SOD-1 protein in ATD, suggesting that it is necessary to increase the amount of protein to obtain adequate levels of SOD activity in ATD patients. Our data do not agree with those reported by Padurariu et al. (2010), who showed decreased serum SOD activity in ATD patients. However, our patients are on an early stage of the illness, as indicated by the mini-mental score. Thus, in the work of Padurariu mini-mental score was 18.5 ± 0.3, whereas in our patients was 23.7 ± 0.9 in men and 20.7 ± 0.6 in women, suggesting that at these early stages, plasma SOD activity is not yet a sensitive biomarker due to the increase in SOD protein found in ATD patients. In any case, most of the studies which demonstrate changes in SOD activity do not analyze serum or plasma, but uses erythrocytes in the assay.

Regarding the significant decrease of GPx activity, it could indicate the failure of the antioxidant system to produce enough GSH to avoid oxidative stress produced by ROS, and the decrease in CAT activity could also indicate the failure in removing H_2O_2. In both cases, the decrease of enzyme activities is not related with the decrease of the amount of the enzymes, as indicated by western blot analysis. Further studies must evaluate the mechanism involved in enzyme activities inhibition (It could be possible that the higher protein oxidation levels found in ATD patients could affect these enzyme activities), or if other forms of GPx other than GPx-1 are affected. All this data strongly suggest that the observed changes are related to the physiopathology of the ATD. Previous studies have suggested contradictory results in relation with the activity of antioxidant enzymes in neurodegenerative disorders. Some researchers reported an increase in the activity of these enzymes (Aybek et al., 2007; Baldeiras et al., 2008; Kawamoto et al., 2005), while others (Fernandes et al., 1999) reported a decrease in the activity. Finally, other authors did not observed any differences (Bourdel-Marchasson et al., 2001; Ceballos-Picot et al., 1996). Our data agree with those recently reported by Padurariu et al. (2010) who have also described a significant decrease in serum GPx activity in mild cognitive impairment (MCI) and ATD patients, and there were no significant differences between genders. In this way, these data have suggested that the increased production of oxygen and nitrogen reactive species in neurodegenerative

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disorders might lead to a rapid consumption of plasma antioxidants. As depleted, the antioxidant systems fail to protect the organism against the oxidative damage, so early ATD patients may have an inadequate antioxidant enzymatic activity that is incapable of responding to increased free radical production, which could lead to the development of the pathological alteration (Baldeiras et al., 2008; Padurariu et al., 2010; Sultana et al., 2008).

Although with the limitations of sample size and the inclusion and exclusion criteria of subjects analyzed in the present report, our results support the idea that circulating levels of enzymatic and non-enzymatic defense system biomarkers may be useful to facilitate the diagnosis of ATD in its earliest stage. Moreover, they are easily accessible through non-invasive techniques and non-expensive procedures, and should be validated as reliable tools to implement in clinical practice alongside other blood tests already available to clinicians as risk factor measurements.

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