Oxidative stress in Alzheimer’s and vascular dementias: masking of the antioxidant profiles by a concomitant Type II diabetes mellitus condition

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Abstract

Oxidative stress is associated with Alzheimer’s (DAT) and vascular (VD) dementias, as well as Type II diabetes mellitus (DIAB) and affected by hypoglycemic therapy. The population (n = 122; males = 60; mean age = 72.57 ± 7.06) consisted of controls (CTR), DAT and VD patients, with (DAT + DIAB, VD + DIAB) and without concomitant DIAB, resulting in six groups where the antioxidant profile was determined: copper–zinc superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), and total antioxidant capacity (TRAP). The results were analyzed using a two-way ANOVA design and Bonferroni statistic. The ANOVAs yielded significant differences between groups for all components of the profile: SOD, \( p = 0.00000006 \); TBARS, \( p = 0.0000012 \); TRAP, \( p = 0.0000003 \). The significance level for comparisons between groups was set at \( \alpha = 0.05 \). The comparisons DIAB vs. CTR, DAT + DIAB vs. DAT, and DIAB demented vs.
1. Introduction

Oxidative stress has been demonstrated in blood profiles of living neurological patients in association with probable dementia of the Alzheimer’s type (DAT) and vascular dementia (VD) among other neurological disorders [1–4], both diseases presenting characteristic antioxidant profiles by means of which it is possible to differentiate and identify the pathologies, with the activity values of the Cu–Zn superoxide dismutase (SOD) acting as a leading and early marker.

The association between the non-insulin-dependent Type II diabetes mellitus (DIAB) and oxidative stress has been widely reported. Antioxidants and free radicals are now regarded as central players in DIAB. The prevalence of DIAB increases with age, up to 12% after 65 years; an increase in oxidative stress and a decrease in antioxidant defenses have been shown in aged individuals [5]. A recent study [6] reports the concept that oxidative stress leads to oxidative injury of dorsal root ganglion neurons, with mitochondrion as a specific target, leading to impaired mitochondrial function and apoptosis, manifested clinically as a predominantly sensory neuropathy. In other reports, an enhanced oxidative stress has been observed as indicated by increased free radical production [7], lipid peroxidation and diminished antioxidant status [8]. DIAB patients also present an enhanced oxidative stress [9] with a significant increase of thiobarbituric acid-reactive substances (TBARS) concentration in parallel with a significant decrease of unsaturated fatty acids concentration, and significantly increased SOD activity, as well as significantly decreased reduced glutathione and α-tocopherol concentrations. Moreover, as an easy tool to predict type II diabetes risk, a practical numerical score has been proposed recently [10].

In this research, the antioxidant profiles comprise measurements of SOD and TBARS in red blood cells (RBC), and total antioxidant capacity (TRAP) in plasma. SOD promotes the conversion of the superoxide free radical anion into oxygen and hydrogen peroxide; this mechanism [11] prevents in normal metabolism the formation of the highly cytotoxic oxygen-derived free radicals. TBARS are substances produced during lipid peroxidation (one of these is the malondialdehyde, MDA) and represent a measure of total free radical potential damage. TRAP, which includes the hydrophilic and/or lipophilic antioxidants, is a measure of the total antioxidant protection.

The simultaneous measurement of all the variables of the antioxidant profile provides information on their correlations allowing the comparison of pathological entities. The study of antioxidant blood profiles in diseased individuals has a potential for being used in understanding the associated pathogenesis and their clinical and biochemical heterogeneity.

Patients showing a demential syndrome, whatever its etiology, constitute an heterogeneous group. Thence, when studying demented patients, it is safer to construct groups as homogenous as possible with regard to characteristics other than those defining the general syndrome. Additionally, not only in dementias heterogeneity is one of the characteristics of the diseases. Also, the DIAB pathology presents a highly associated heterogeneity [12].

In this prospective and transversal research, the objective was to study demented patients using DIAB as a second criterion, including oral hypoglycemic therapy drugs known for their action on the antioxidant variables. The antioxidant profiles of patients and controls were studied, testing the following hypotheses: (a) Oxidative stress might be linked with DIAB and/or drugs used in living patients; (b) Oxidative stress in DIAB patients might be closer to DAT and VD patients since DIAB is frequently associated with both dementias; (c) The demented condition could be recognized in the antioxidant profiles of patients with DIAB.

2. Materials and methods

2.1. Patients and controls

The initial population of 130 subjects consisted of healthy controls (CTR), DIAB patients, and demented patients of the DAT and VD types with and without associated DIAB. CTR subjects were selected by age and sex to reflect the general gender and age distribution of the diseased groups. Outpatients and controls were from Caucasian origin recruited from the Neurology Service and the Diabetes Unit of the Hospital Sirio-Libanés, Hospital General de Agudos Juan A. Fernández and the FACENE.

Patients and controls were included in the study accordingly with accepted neurological criteria for each group: DAT patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer’s Disease Association and Related
Disorders criteria for a clinical diagnosis of probable DAT (NINCDS-ADRDA) [13]; VD patients according to the NINDS-AIREN criteria [14]; DIAB patients using the criteria revised by the expert group of the American Association of Diabetes (ADA) and the World Health Organization (WHO) [15]; non-demented control subjects were defined using the American Psychiatric Association DSM IV criteria [16].

Patients suffering from systemic or other neurological disorders making diagnostics uncertain were excluded, i.e. head trauma, seizures, uncontrolled hypertension, mental retardation, psychosis or depression, etc. All subjects underwent neurological, psychiatrical, physical examination and comprehensive sets of neurological tests, and were recruited provided that they had not a history of smoking in the last 5 years. VD patients were studied with the Mattis scale [17]. DAT patients were studied with the ADAS scale [18]. Functional assessment for all patients was conducted using CDR [19]. Depression was measured in all patients with the Hamilton test [20].

Since being recruited and until data analysis (a period of 17 months for the first recruited subject), all subjects were controlled every other month. A total of eight subjects were excluded because of various reasons (tumors, infections, death, etc.).

Mean ± standard deviation for the onset time were as follows: for DAT patients 6.52 ± 1.25 (range: 4–9) years; for VD patients 5.64 ± 1.87 (range: 2–7) years; for DIAB patients 10.41 ± 3.85 (range: 4–21) years. All patients with demential syndromes present CDR stages into the range between 1 and 2: for DAT, 48% and 52%; for DAT + DIAB, 43% and 57%; for VD, 40% and 60%; for VD + DIAB, 37% and 63%; respectively. Within the DIAB population, 40% were on sulfonylurea medication (72.73%); 27 (48.10%) on glibenclamide, 8 (14.54%) on glimepiride, and 5 (9.09%) on glicazide; 7 were on biguanide medication (12.73%); 4 were on α-glucosidase inhibitor medication (7.27%); and 4 received no medication but only diet (7.27%).

2.2. Blood sampling

Small volumes of venous blood were obtained with written informed consent from healthy volunteers and patients.

Each subject contributed one sample, which was heparinized and processed; all the laboratory determinations were run in duplicate and the mean was used. As in previous studies, the analysis of the differences of the duplicates indicated that this source of variability is nonsignificant [2].

Heparinized venous blood samples were washed with NaCl solution (0.1314 mol/l) and centrifuged three times at 3000 × g for 10 min at room temperature. The plasma was used for TRAP assay; white cells were discarded by aspiration. RBC were used for enzymatic assays of TBARS and SOD.

2.3. SOD assay

RBC from 5 ml blood samples were washed with bidistilled water and hemolyzed using the osmotic shock technique. Hemoglobin was separated by centrifugation from chloroform–ethanol extracts and discarded; the remaining solution was assayed for SOD.

The SOD activity was determined measuring the ability of RBC’s supernatant to inhibit the autoxidation of epinephrine at pH 10.2, 30 °C [21]. One unit of SOD activity was defined as the inhibition of the epinephrine oxidation rate by 50% [22].

The technique used was previously reported [2]. The protein concentration was determined with the Folin reagent [23], using bovine serum albumin as standard. All reactives used were of analytical grade and were employed in assays without further purification. Activity is expressed in U SOD/mg protein.

2.4. Thiobarbituric acid-reactive substances (TBARS) assay

The method starts with RBC (200 µl) and measure the fluorescence (excitation: 515 nm, emission: 555 nm) in the alcoholic phase of the colored complex formed. The calibration curve was constructed using 1,1,3,3-tetrametoxipropene as standard [24]. The results are expressed in nmol MDA/ml plasma.

2.5. TRAP assay

The plasma antioxidant capacity was measured by chemiluminescence. This method detects the hydrosoluble and/or the liposoluble antioxidants present in plasma [25]. The reaction consisted in 2,2′-azobis(2 amidino-propane, ABAP) and luminol; ABAP is a source of free radicals which reacts with luminol yielding chemiluminescence, which is measured with a liquid scintillation counter in the out-of-coincidence mode [26,27]. The addition of plasma decreases chemiluminescence to basal levels for a period proportional to the charge of antioxidants in plasma (TRAP) until luminol radicals are regenerated. The system is calibrated with Trolox (vitamin E hydrosoluble analog). The results were expressed in µM Trolox.

2.6. Chemicals

The 2-2′-azobis(2 amidino-propane) was from Poliscience, Warrington, PA, USA. All other chemicals were obtained from Sigma (St. Louis, MO).

2.7. Statistical analysis

A two-way analysis of variance (ANOVA) [28] was performed and the Bonferroni statistic [28] employed to compare mean values of groups of patients and controls using an overall significance level α = 0.05, which
resulted in a critical value of \( t = 2.5233 \); and a nonparametric technique to study, within groups, the effect of treatment with sulfonylureas.

For display and comparison, all data were standardized subtracting from each individual measurement the overall mean (calculated across all treatments) and dividing by the overall standard deviation.

### 3. Results

The total population of 122 subjects (eight subjects were excluded from the initial population of 130, see Section 2.1), 60 males (49.18%), mean age 72.57 years (standard deviation 7.06); resulted in six groups: 19 CTR subjects, 18 DIAB patients, 29 nondiabetic DAT patients, 19 diabetic DAT patients (DAT + DIAB), 19 nondiabetic VD patients, and 18 diabetic VD patients (VD + DIAB).

Basic statistics of the six experimental groups are shown in Table 1, together with the means and standard deviations obtained for TBARS, SOD and TRAP. It should be noted that mean age in the DIAB group was smaller than in the remaining. Nevertheless, a large degree of overlap in their age distributions occurs.

Fig. 1 presents the standardized mean profiles of the six experimental groups. While the CTR and DIAB antioxidant profiles appear to be similar, those of DAT and VD groups are widely different from their diabetic counterparts.

Fig. 2 was constructed to show the differences in the antioxidant profiles observed between: DIAB–CTR groups, (DAT + DIAB)–DAT groups, and (VD + DIAB)–VD groups. The non-demented difference (DIAB–CTR) remains close to zero, while the VD and DAT groups show large differences in TBARS and SOD. Nevertheless, TRAP remains approximately the same.

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>CTR, ( n = 19 ) (12 M/7 F)</th>
<th>DIAB, ( n = 18 ) (10 M/8 F)</th>
<th>DAT, ( n = 29 ) (6 M/23 F)</th>
<th>DAT + DIAB, ( n = 19 ) (7 M/12 F)</th>
<th>VD, ( n = 19 ) (11 M/8 F)</th>
<th>VD + DIAB, ( n = 18 ) (13 M/5 F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD*</td>
<td>8.879</td>
<td>9.972</td>
<td>16.090</td>
<td>10.053</td>
<td>15.753</td>
<td>12.072</td>
</tr>
<tr>
<td>TBARS*</td>
<td>2.954</td>
<td>2.894</td>
<td>3.985</td>
<td>3.295</td>
<td>3.492</td>
<td>3.087</td>
</tr>
<tr>
<td>TRAP*</td>
<td>451.579</td>
<td>411.778</td>
<td>283.621</td>
<td>316.362</td>
<td>361.368</td>
<td>377.944</td>
</tr>
<tr>
<td>AGE†</td>
<td>73.89</td>
<td>67.44</td>
<td>73.35</td>
<td>73.21</td>
<td>74.32</td>
<td>72.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CTR, ( n = 19 ) (12 M/7 F)</th>
<th>DIAB, ( n = 18 ) (10 M/8 F)</th>
<th>DAT, ( n = 29 ) (6 M/23 F)</th>
<th>DAT + DIAB, ( n = 19 ) (7 M/12 F)</th>
<th>VD, ( n = 19 ) (11 M/8 F)</th>
<th>VD + DIAB, ( n = 18 ) (13 M/5 F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std dev.</td>
<td>1.792</td>
<td>3.624</td>
<td>3.759</td>
<td>3.619</td>
<td>5.811</td>
</tr>
<tr>
<td>Std dev.</td>
<td>0.488</td>
<td>0.537</td>
<td>0.793</td>
<td>0.735</td>
<td>0.753</td>
</tr>
<tr>
<td>Std dev.</td>
<td>64.524</td>
<td>122.911</td>
<td>85.868</td>
<td>70.442</td>
<td>98.032</td>
</tr>
</tbody>
</table>

\* [SOD] is expressed in U SOD/mg protein.

\* [TBARS] is expressed in nmol MDA/ml plasma.

\* [TRAP] is expressed in \( \mu M \) Trolox.

\* [AGE] is expressed in years; \( n \) is the total number of subjects in each group.

For display and comparison, all data were standardized subtracting from each individual measurement the overall mean (calculated across all treatments) and dividing by the overall standard deviation.
ANOVA results for each component of the antioxidant system are shown in Table 2, together with the Bonferroni statistics calculated for the planned comparisons: DIAB vs. CTR, DAT + DIAB vs. DAT, VD + DIAB vs. VD, and diabetes effect on non-demented vs. diabetes effect on demented subjects. The proportion of males in each comparison was as follows: 0.55 vs. 0.63, 0.36 vs. 0.21, 0.72 vs. 0.58, and 0.55 vs. 0.54, respectively.

The components of the antioxidant system have shown highly significant differences between the experimental groups: $p = 0.00000006$ for SOD, $p = 0.0000012$ for TBARS, $p = 0.0000003$ for TRAP; while the $p = 0.01947$ associated with age resulted slightly significant.

The comparisons between groups (Bonferroni statistic) were calculated at an overall significance level $\alpha = 0.05$. Accordingly with Bonferroni’s method, an overall significance level of 0.05 is achieved when individual tests are significant at the 0.0125 level; the corresponding critical value with the present design (degrees of freedom, $v = 116$) is $t_c = \pm 2.5233$.

The comparison of DIAB vs. CTR groups resulted nonsignificant for TBARS, but significant for the other

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Table 2

<table>
<thead>
<tr>
<th>SOD</th>
<th>TBARS</th>
<th>TRAP</th>
<th>AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.478</td>
<td>3.346</td>
<td>359.852</td>
<td>72.500</td>
</tr>
<tr>
<td>5.242</td>
<td>0.785</td>
<td>109.332</td>
<td>7.207</td>
</tr>
<tr>
<td>1055.879</td>
<td>20.088</td>
<td>418,348.987</td>
<td>685.507</td>
</tr>
<tr>
<td>2296.985</td>
<td>55.018</td>
<td>1,039,974.357</td>
<td>5650.993</td>
</tr>
<tr>
<td>3352.865</td>
<td>75.107</td>
<td>1,458,323.344</td>
<td>6336.500</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>116</td>
<td>116</td>
<td>116</td>
<td>116</td>
</tr>
<tr>
<td>10.665</td>
<td>8.471</td>
<td>9.333</td>
<td>2.814</td>
</tr>
</tbody>
</table>

| Probability | 0.00000006 | 0.00000012 | 0.00000003 | 0.01947 |

Bonferroni statistic

<table>
<thead>
<tr>
<th>SOD</th>
<th>TBARS</th>
<th>TRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIAB vs. CTR</td>
<td>$-2.547^*$</td>
<td>0.920</td>
</tr>
<tr>
<td>DAT + DIAB vs. DAT</td>
<td>18.028*</td>
<td>13.095*</td>
</tr>
<tr>
<td>VD + DIAB vs. VD</td>
<td>9.006*</td>
<td>6.196*</td>
</tr>
<tr>
<td>DIAB NON DEM. vs. DIAB DEM.</td>
<td>$-9.859^*$</td>
<td>$-5.133^*$</td>
</tr>
</tbody>
</table>

*Difference found significant at the 0.05 level (see Results for details).
studied variables. The comparison of DAT + DIAB vs. DAT yielded significant differences for the three studied components of the antioxidant system. The comparison of VD + DIAB vs. VD groups resulted in significant differences for TBARS and SOD, nonsignificant for TRAP.

The effect of the demented condition in DIAB patients, i.e. the comparison of DIAB demented vs. DIAB non-demented groups, yielded significant differences for all the variables. This effect is depicted in Fig. 2, where the plotted differences in the demented groups present similar profiles, while the difference DIAB – CTR shows a very different shape.

The effect of the different hypoglycemic treatments was tested using a rank test. Within the non-demented DIAB group, patients treated with sulfonylureas showed a significant decrease in TBARS against other therapies; in the DAT + DIAB group, patients under sulfonylureas treatment presented significantly lower levels of SOD; no differences were found within the VD + DIAB group, nor between the different sulfonylureas treatments employed (glibenclamide, glimepiride and glicazide) [29].

No correlations were found in each group between age and the variables of the antioxidant system TBARS and TRAP, other than the previously reported for SOD and age [1–4,30–32], where two different linear regressions of the SOD activity as a function of the age of DAT patients are found below and above approximately 70 years. Additionally, these investigations have shown no association between sex and antioxidant profiles in demented (DAT and VD) patients.

4. Discussion

DAT and VD patients with a concomitant DIAB condition (DAT + DIAB and VD + DIAB groups) show SOD and TBARS values closer to non-demented subjects (Fig. 1), thus reducing the large effect of the dementia in these variables. The resulting overlap in their distributions masks the clear-cut difference in the antioxidant profiles already reported for nondiabetic demented patients.

It has been suggested that oxidative stress may play an important role in the pathogenesis of diabetic complications. The changes observed in one report [33], which is focused on oxidation reactions and the prevention of diabetic complications, suggest a significant imbalance in the antioxidant system of the skeletal muscle of rabbits with alloxan-induced diabetes.

Consistent with the interpretation that a generalized oxidative stress associated with aging may contribute to the development of age-related pathologies such as DIAB, an increased oxidative damage in Type I and Type II DIAB as well as deficits in the antioxidant defense enzymes and vitamins was reported [34], showing that the oxidation of glucose leads to hydrogen peroxide and reactive ketoaldehydes that participate in the formation of glycated proteins, another source of free radicals. Together with the depletion of cellular antioxidant defense mechanisms, these end-products play a major role in the pathogenesis of diabetic vascular complications: endothelium-dependent vasodilation and inactivation of endothelium-derived releasing factors [34]. Also, DIAB in an older population is associated with increased basal oxidative stress accentuated by hyperglycemic challenge [35], and autodislation of glucose was reported as one of the main sources of free radicals in diabetes [36].

In a recent investigation [37], it was hypothesized that because reactive oxygen species (ROS) are produced by oxidative phosphorylation during anaerobic glycolysis, via the Schiff reaction during glycation, via glucose autoxidation, and via hexosamine metabolism under supraphysiological glucose concentrations, chronic oxidative stress is an important mechanism for glucose toxicity. Clinically, consideration of antioxidants as adjunct therapy in type II diabetes is warranted because of the many reports of elevated markers of oxidative stress in patients with this disease.

The risk of developing vascular diseases in DIAB patients is significantly increased: its etiology may involve oxidative damage by free radicals, and protection against such damage can be offered by antioxidant systems [38]; in this investigation, it was reported that a significant increase in MDA concentration was found in DIAB patients compared to controls ($p < 0.005$), together with a significant drop in plasma thiol groups ($p < 0.05$). No significant difference was observed in the total antioxidant capacity. It is interesting to note that the results reported here are opposed to the above with respect to TBARS and TRAP, but confirm the presence of oxidative stress in the comparison between CTR and DIAB patients.

Since no particular substance has been found in association with DAT and VD diseases, as it was the case with glucose in DIAB, causal mechanisms explaining the association found between oxidative stress and dementia are rather speculative and involve ROS [39], elevation of hydrogen peroxide synthesis [40], and advanced glycation end-products [41].

It should be taken into account that an effect on living patients of hypoglycemic treatments—particularly sulfonylureas—in the components of the antioxidant profile was detected in this study. Under the present experimental design, it is not possible to isolate the effect of drugs from the effect of DIAB itself. Consequently, the present results cannot be referred solely to the effects of the disease, because they are compounded with the drugs used.

Finally, in relation with the hypotheses tested in the present study, it might be concluded that: (a) In DIAB living patients, a link exists between the oxidative stress and the disease, more evident in demented patients; (b) Antioxidant profiles of demented DIAB patients (DAT + DIAB and VD + DIAB) might be hidden by the drug used and cannot be directly related with any of the studied
dementias; (c) The lowered TBARS and SOD values in demented DIAB patients as compared with demented (DAT and VD) suggest that the diagnostic value of the antioxidant profile might be masked by the concomitant DIAB condition and/or the drugs used.

Acknowledgements

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