Neurochem Res (2009) 34:2122–2132
DOI 10.1007/s11064-009-9997-5

ORIGINAL PAPER

Systemic Oxidative Stress Associated with the Neurological Diseases of Aging

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Accepted: 9 May 2009 / Published online: 2 June 2009

Abstract Markers of oxidative stress were measured in blood samples of 338 subjects (965 observations): Alzheimer's, vascular dementia, diabetes (type II) superimposed to dementias, Parkinson's disease and controls. Patients showed increased thiobarbituric acid reactive substances (+21%; $P < 0.05$), copper-zinc superoxide dismutase (+64%; $P < 0.001$) and decreased antioxidant capacity (−28%; $P < 0.001$); pairs of variables resulted linearly related across groups ($P < 0.001$). Catalase and glutathione peroxidase, involved in discrimination between diseases, resulted non-significant. When diabetes is superimposed with dementias, changes resulted less marked but significant. Also, superoxide dismutase resulted not linearly correlated with any other variable or age-related (pure Alzheimer's peaks at 70 years, $P < 0.001$). Systemic oxidative stress was significantly associated ($P \ll 0.001$) with all diseases indicating a disbalance in peripheral/adaptive responses to oxidative disorders through different free radical metabolic pathways. While other changes—methionine cycle, insulin correlation—are also associated with dementias, the responses presented here show a simple linear relation between prooxidants and antioxidant defenses.

Keywords Free radicals · Oxidative stress · Aging · Alzheimer's disease · Vascular dementia · Parkinson disease · Type II diabetes mellitus

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Introduction

As an increased neuronal production of the products of the partial reduction of oxygen reactive oxygen species (O_2^-, H_2O_2, $HO^\bullet$) and of reactive nitrogen species ($NO^\bullet$, $ONOO^-$) occurs in neurodegenerative diseases and neurological disorders apparently associated to a systemic oxidative stress (SOS) it becomes an interesting topic to describe variations in its markers. The brain and the central nervous system are known to have an important and constant energy demand, i.e., to require an important and constant supply of substrates and molecular oxygen. Brain oxygen consumption is estimated at 20% of the total oxygen uptake in basal metabolic conditions. Mitochondria, the subcellular site of oxygen uptake, are recognized as the primary source and also as the target of oxidizing free radicals [1, 2].

Oxidizing free radicals include the products of the partial reduction of oxygen and of reactive nitrogen species. The rationale for oxidative stress can be described conceptually at the molecular level as an increase in the rate of generation of any of the species that are primarily produced in the biological system by enzyme-catalyzed reactions. They result in an increased steady-state level of the free radicals species that leads to an increased rate of generation of the downstream species produced by non-enzymatic second order reactions, finally leading to an increased rate of lipid peroxidation among other oxidative processes. Lipid peroxidation reactions generate secondary products that are markers of oxidative damage and of oxidative stress [1, 3].

The currently recognized markers, *consensum scholarum*, for systemic oxidative stress in human subjects are the plasma levels of thiobarbituric acid reactive substances (TBARS), total reactive antioxidant potential (TRAP), α-tocopherol and ascorbic acid. The increase in TBARS results from augmented levels of systemic and neuronal hydroperoxides that lead to an increment in lipid peroxidation. The decreased TRAP indicates reduced total levels of antioxidant substances [1, 4].

Moreover, an increase in the activity of antioxidant enzymes, i.e., Cu–Zn superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), in red blood cells (RBC) have been regarded as markers of systemic oxidative stress, since the up-regulation of antioxidant enzyme expression was considered as an adaptive response to the oxidative stress situation. Accordingly to both criteria—plasmatic and antioxidant enzymes markers—a significantly systemic oxidative stress has been observed in Alzheimer's patients (AD), in non-degenerative vascular dementia (VD) and in Parkinson's disease (PD) [5–8].

In humans, the antioxidant enzymes SOD, CAT, and GPx, have complementary functions and act cooperatively and synergistically with other substances (e.g., α-tocopherol) to achieve adequate levels of antioxidant activity in the body. Then, it is reasonable to consider the antioxidant activities in an integrated form that was defined as the antioxidant profile [8].

The association with a systemic oxidative stress was also found in patients with type II diabetes mellitus (DIAB) alone, and when diabetes is concomitant with dementia (AD + DIAB, VD + DIAB); notwithstanding that the changes in the variables are less marked, they are still significant [9].

To deal with the intrinsic heterogeneity within groups of patients with dementia it is necessary to select subjects as homogeneous as possible with regards to characteristics other than those defining the general pathology. Additionally, not only the dementias, but also the diabetes pathology presents a highly associated heterogeneity [10].

With the integration of a large number of patients/subjects and observations from several protocols it becomes possible to test hypothesis, such as described below, involving many groups and variables by means of robust and conservative statistical methods.

The overall relationships between oxidative markers were studied both at the patient level and considering their changes across several independently recruited groups, bearing in mind the integrated view of the oxidative stress phenomenon as described by the Sies scale [11]. The hypothesis tested here, by means of a large number of patient/subjects, was that the balance between prooxidants and defense species, related with neurological diseases, might be interpreted as the result of simple counteracting effects producing oxidative stress and/or oxidative damage.

Materials and Methods

Patients and Controls

The 338 human subjects (182 females/156 males; mean age ± standard deviation: 71.4 ± 8.6 years) consisted of healthy controls (C), Alzheimer's disease (AD), vascular dementia (VD) and Parkinson's disease (PD) patients; moreover, diabetic (type II) patients (DIAB condition) with and without superimposed dementias were included. Controls were healthy subjects; outpatients and controls were Caucasian, recruited from the Neurology Service of Hospital Sirio-Libanés and from the Diabetes Unit of Hospital Juan Fernández, Buenos Aires. Exclusion criteria were neurological disorders making diagnostic uncertain such as head trauma, seizures, uncontrolled hypertension, mental retardation and psychosis or depression. All subjects underwent neurological, psychiatric and physical examination, and a history of not smoking in the last 5 years.

Functional cognitive assessment conducted using the clinical demential rating (CDR) [12] and the global deterioration scale (GDS) [13]. Depression was evaluated in all patients with the Hamilton test [14].

Since recruitment and until data analysis (a period of up to 17 months) all subjects were controlled every month over a period of at least 6 months. A total of 38 subjects were excluded because of various reasons (tumors, infections, death, less than six visits, etc.). Seven groups were formed:

AD Group: One hundred-twelve patients fulfilling 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 AD (NINCDS–ADRDA) [15], and studied with the Alzheimer's disease assessment scale (ADAS) [16]. Patients were included with a GDS stage of 3–5. No patient presented vascular lesions on computed tomography (CT) or magnetic resonance images (MRI) scanning.

VD Group: Fifty-seven patients fulfilling the criteria for the diagnosis of ischemic VD according to the NINDS–AIREN criteria [17], and studied with the Mattis scale [18]. All patients had supratentorial multiinfarction images on CT or MRI scanning, and a CDR stage of 1–2.

PD Group: Fifteen patients with, at least, two cardinal symptoms of idiopathic PD according to clinical criteria [19], a Hoehn and Yahr stage of 2–4 [20], and a history of positive response to levodopa therapy. No patient presented vascular lesions on CT or MRI scanning.

Diabetic patients Group (DIAB condition): Eighteen patients according to the revised criteria of the expert group of the American Association of Diabetes and the World Health Organization [21].

AD + DIAB Group: Nineteen patients fulfilling both [15, 16] and [21] criteria.

VD + DIAB Group: Eighteen patients fulfilling both [17, 18] and [21] criteria.

Control Group: Eighty control subjects meeting the American Psychiatric Association DSM IV criteria [22], and without neurological symptoms, selected by age and sex to reflect the gender and age distribution of the patients groups. No subject presented lesions on CT or MRI scanning.

Mean ± standard error for the onset time were for AD patients 7.3 ± 0.8 (range: 5–9) years; for VD patients 6.3 ± 1.0 (range: 3–8) years; for PD patients 6.1 ± 1.1 (range: 4–9) years; for DIAB patients 10.4 ± 1.9 (range: 4–21) years. All patients with dementia syndromes presented mild and moderate stages, into the range 1 and 2 of CDR: for AD, 42 and 58%; for AD + DIAB, 43 and 57%; for VD, 38 and 62%; for VD + DIAB, 37 and 63%; respectively.

Patients suspended the intake of drugs, which have some proved effects on the measured variables. Recorded drugs were digital, aspirin, levodopa, enalapril and diazepam. Within the DIAB population ($n = 55$), 40 patients (72.8%) were on sulfonylurea medication (27 on glibenclamide, 8 on glimepiride, and 5 on glicazide), 7 patients (12.7%) were on biguanide medication, 4 patients (7.2%) were on α-glucosidase inhibitor medication, and 4 patients (7.2%) received no medication but only diet.

Blood Sampling

Venous blood was obtained with informed consent from controls and patients, with one sample per subject and the laboratory determinations in duplicate. Heparinized blood samples were centrifuged at 3,000*g* for 10 min at room temperature. Plasma was used for TBARS and TRAP assays and red blood cells (RBC) for determination of enzymatic activities of SOD, CAT and GPx.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Plasma (1 ml) was added with 0.1% butyl-hydroxyl-toluene, 1 ml 5% trichloroacetic acid, and 0.7% 2-thiobarbituric acid. The samples were heated for 15 min in a water bath at 100°C, cooled at room temperature and extracted with 3 ml *n*-butanol. After agitation and centrifugation, the fluorescence was determined in the butanol phase using 515 nm for excitation and 555 nm for emission (HITACHI F-3010 spectrofluorometer). The calibration curve was done with 1,1,3,3-tetrametoxi-propane as standard [23]. The results are expressed in nmol malonaldheyde (MDA)/ml plasma.

Plasma Antioxidant Capacity (TRAP) Assay

Plasma antioxidant capacity was determined by the ABAP method [24, 25] that integrative measures hydrosoluble and liposoluble antioxidants in plasma [26]. The reaction medium consisted in 20 mM 2,2′-azobis (2 amidinopropane, ABAP) in phosphate buffer 50 mM (pH 7.4), and 40 μM luminol: ABAP is a source of free radicals which reacts with luminol yielding chemiluminescence, that was determined at room temperature with a liquid scintillation counter (LKB WALLAC 1209 RACK BETA) in the out-of-coincidence mode. The addition of 10–20 μl of plasma decreases the initiation of the chemiluminescence burst for a period (induction time, σ) proportional to the plasma content of antioxidants. The system is calibrated with the (vitamin E hydrosoluble analog) Trolox and allows the assessment of TRAP values as equivalents to Trolox concentrations. The TRAP values are obtained employing the following equation:

$$\text{TRAP}\,(\mu\text{M Trolox}) = D \cdot \sigma S/\sigma T$$

where D is a dilution factor, σS are the sample induction time and σT is the induction time in the presence of 1 μm Trolox. Results are expressed as equivalent to Trolox concentrations.

Cu–Zn Superoxide Dismutase (SOD) Activity Assay

RBC from 5 ml blood samples were hemolyzed with 5 ml of distilled water and hemoglobin was separated by addition of a 2:1 volume of chloroform–ethanol (3:5) at 0°C and centrifugation [6, 27]. The remaining solution was assayed for SOD by measuring the ability of the extract to inhibit the auto-oxidation of epinephrine at pH 10.2 and at 30°C [28]. One unit of SOD activity is defined as the inhibition of the epinephrine oxidation rate by 50% [29], and corresponds to 32 pmol of Cu–Zn SOD. The absorption changes were measured at 480 nm (BECKMAN DU 650 spectrophotometer). Activity is expressed in U_{SOD}/mg protein.

Catalase (CAT) Activity Assay

RBC (1 ml) was hemolyzed with equal volume of distilled water at 4°C and diluted 1:500 in 50 mM phosphate buffer (pH 7.0). CAT activity was determined by addition of 10 mM H_2O_2 and the absorbance decrease was spectrophotometrically measured at 240 nm and expressed as the pseudo-first order reaction constant (K')/ml RBC. The considered second order reaction constant was $k' = 4.6 \times 10^7\ \text{M}^{-1}\ \text{s}^{-1}$ [30].

Glutathione Peroxidase (GPx) Activity Assay

RBC (1 ml) was treated with 9 mM KCN and 1.2 mM $K_3[Fe(CN)_6]$ to convert hemoglobin into cianomethemoglobin. The sample was incubated 10 min at 37°C with 1 mM EDTA, 100 mM K_3PO_4, 10 mM NADPH, 50 μl GSH 100 mM, 10 μl azide 40 mM, and 10 μl glutathione reductase (10 U/ml); then 50 μl tert-butyl-hydroperoxide (BOOH) 10 mM was added and the absorbance was spectrophotometrically measured at 340 nm ($\varepsilon_{340} = 6.23\ \text{nM}^{-1}\ \text{cm}^{-1}$). Activity is expressed as reduced glutathione (GPx) equivalent: μM GPx/ml RBC [31].

Protein Concentration Assay

The protein concentration was determined with the Folin reagent [32] using bovine serum albumin (grade III) as standard.

Chemicals

The 2-2′-azobis (2 amidinopropane) was from Poliscience, Warrington, PA, USA. All the other chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical Analysis

Differences between groups were tested simultaneously on the five measured variables (TBARS, TRAP, SOD, CAT, and GPx) by means of a multivariate one-way analysis of variance model (MANOVA) using Roy's statistic (largest characteristic root) [33] to test the usual null hypotheses ($\mu_{i,\,j} = \mu_{l,\,k}$).

One-way analyses of variance (ANOVA) [34] were performed on separate variables in the groups, and the Bonferroni's statistic [34] was used to test the group differences against the control group. The overall significance level of $\alpha = 0.05$ was achieved with critical "t" (t_c) values 2.408, 2.401, and 2.393 for TBARS, TRAP, and SOD, respectively. In the patients with a superimposed diabetic condition the critical value was $t_c = 2.435$.

The associations between the five measured variables were analyzed by means of Spearman's coefficient of correlation. A non-parametric technique was applied to study, within each group of the diabetic condition, the effect of treatments with sulfonylurea.

Results

The plasma levels of products of the lipid peroxidation process, measured as TBARS, and of integrated hydrophilic and hydrophobic antioxidants, measured by the TRAP assay, as well as the erythrocyte enzymatic activities of SOD, CAT, and GPx were determined in elder healthy subjects and in patients with Alzheimer's disease, vascular dementia, and Parkinson disease. Demographical and laboratory data are given in Table 1, and comprised 264 subjects and 743 observations. Patients with the mentioned neurological diseases showed significantly increased (with respect to controls) plasma levels of TBARS (24, 20, and 19%, respectively), significantly decreased plasma TRAP capacity (32, 25, and 28%, respectively) and significantly increased SOD activity in red blood cells (73, 63, and 55%, respectively) with unchanged erythrocyte CAT and GPx activities (Table 1). The differences in plasma TBARS and TRAP levels and in erythrocyte SOD between the control healthy subjects and the neurological patients with either Alzheimer's disease, vascular dementia or Parkinson disease are highly significant, specially after considering that

Table 1 Systemic oxidative stress in neurological diseases. Demographical and laboratory data

Group/Age	TBARS[a]	TRAP[b]	SOD[c]	CAT[d]	GPx[e]
Healthy controls (C)/68.4 ± 1.4 years	2.91 ± 0.08	410 ± 19	10.24 ± 0.28	45.8 ± 3.9	1.9 ± 0.2
$n_T = 80$ (42F/38M)	$n = 33$	$n = 46$	$n = 80$	$n = 25$	$n = 14$
Alzheimer's disease (AD)/72.1 ± 0.6 years	3.61 ± 0.13***	277 ± 12***	17.75 ± 0.47***	43.9 ± 3.0	1.8 ± 0.1
$n_T = 112$ (72F/40M)	$n = 51$	$n = 68$	$n = 112$	$n = 37$	$n = 22$
Vascular dementia (VD)/73.1 ± 0.9 years	3.50 ± 0.12**	309 ± 18***	16.69 ± 0.61***	39.4 ± 3.8	2.1 ± 0.2
$n_T = 57$ (28F/29M)	$n = 34$	$n = 48$	$n = 56$	$n = 27$	$n = 15$
Parkinson's disease (PD)/71.3 ± 2.2 years	3.46 ± 0.18*	295 ± 26**	15.83 ± 0.57***	49.5 ± 3.4	2.1 ± 0.3
$n_T = 15$ (8F/7M)	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$

Results are expressed as mean ± standard error

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, as compared with controls

[a] TBARS are expressed as nmol MDA/ml plasma. [b] TRAP is expressed as μM Trolox. [c] SOD is expressed as U_{SOD}/mg protein. [d] CAT is expressed as k'/ml RBC. [e] GPx was expressed as reduced glutathione (GPx) equivalent: μM GPx/ml RBC. n_T is the total number of subjects in each group. n is the number of observations. F females, M males

The one-way MANOVA model was fitted to the 66 subjects (22 AD, 15 VD, 15 PD and 14 C) with simultaneous observations of the five variables TBARS, TRAP, SOD, CAT, and GPx, yielding a highly significant result ($\theta = 0.577$, $S = 3$, $M = 0.5$, $N = 28$ and $P < 0.001$). CAT and GPx were not correlated with the direction defined by the largest characteristic root ($\lambda = 1.366$) and were left out of the analysis. The direction expressed by director's cosines was: 0.345 TBARS, −0.565 TRAP, and 0.736 SOD (0.115 for CAT and 0.083 for GPx). For TBARS, TRAP, and SOD alone, the values of director's cosines showed small changes: 0.341, −0.569, and 0.748, respectively. The Kruskal–Wallis test yielded a highly significant result ($H = 32.58$, $P \ll 0.001$) for the association of all clinical conditions against controls and the variables TBARS, TRAP, and SOD

Further analysis was conducted by one-way ANOVA's followed by Bonferroni's test yielding highly significant F values for the differences in TBARS (133 observations), TRAP (177 observations) and SOD (263 observations), (v = degrees of freedom): TBARS ($F = 6.122$, $v = 3/129$, $P = 3.61 \times 10^{-4}$), TRAP ($F = 12.943$, $v = 3/173$, $P = 1.49 \times 10^{-7}$), SOD ($F = 54.679$, $v = 3/259$, $P = 5 \times 10^{-14}$); while CAT (104 observations, $F = 1.057$, $v = 3/100$, $P = 0.186$) and GPx (66 observations, $F = 1.164$, $v = 3/62$, $P = 0.169$) yielded a non-significant F and P values

According to Bonferroni's method, a significance level of 0.05 is achieved when values are significant at the 0.00833 level; the corresponding critical value with the present design is $t_c = \pm 2.408$ for TBARS, ±2.401 for TRAP and ±2.393 for SOD (small differences in t_c are due to different v in each variable). The comparisons C vs. AD, C vs. VD, and C vs. PD yielded the following t values: for TBARS ± 4.129, ±3.191, and ±2.463; for TRAP: ±6.067, ±4.245, and ±3.350; for SOD: ±12.362, ±8.920, and ±4.784, respectively

the conservative Bonferroni's statistical test gave P values well below the usual 0.05 threshold.

The determinations of the oxidative markers are comparable with levels reported in the literature, *inter alia*, for plasma TBARS [35–37] and TRAP [38, 39]; and for erythrocyte SOD [36–38, 40–42], CAT [36, 37, 42, 43] and GPx activities [36, 37, 43, 44].

The plasma levels of lipid peroxidation products (TBARS) and total antioxidants (TRAP) showed a significant linear negative correlation ($r = -0.989$, $P < 0.001$, Fig. 1). Moreover, there is also a significant positive linear correlation between plasma TBARS and erythrocyte SOD ($r = 0.998$, $P < 0.001$, Fig. 2). The linear correlation described between the three variables also include a significant negative linear correlation between plasma TRAP and erythrocyte SOD ($r = -0.980$, $P < 0.001$, not shown), which is implied by the other two relationships.

The same indicators that were recognized as markers of neurological diseases in aged humans, i.e., plasma TBARS and TRAP and erythrocyte SOD, were assayed in neurological patients (only in patients with Alzheimer's disease and with vascular dementia) with the condition of

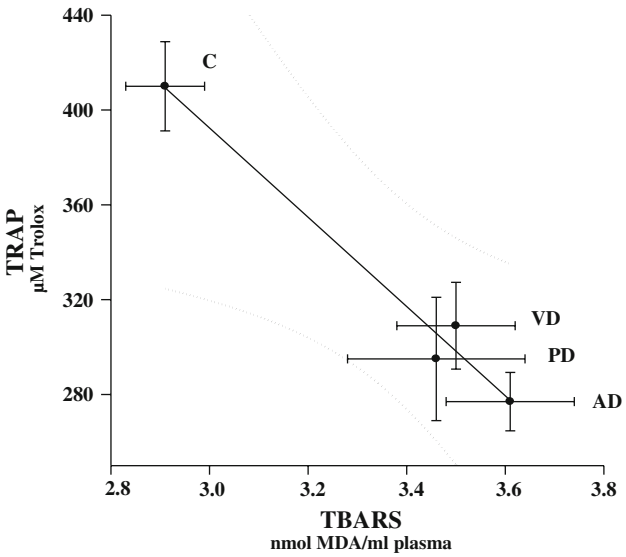

Fig. 1 Correlation between plasma TBARS and plasma TRAP values. Mean values (*points*) and standard error (*bars*), *solid line* is the linear regression ($r = -0.989$), *dotted lines* the 99% confidence intervals. Data from Table 1. *C*, healthy controls; *AD*, Alzheimer's disease patients; *VD*, vascular dementia patients; *PD*, Parkinson's disease patients

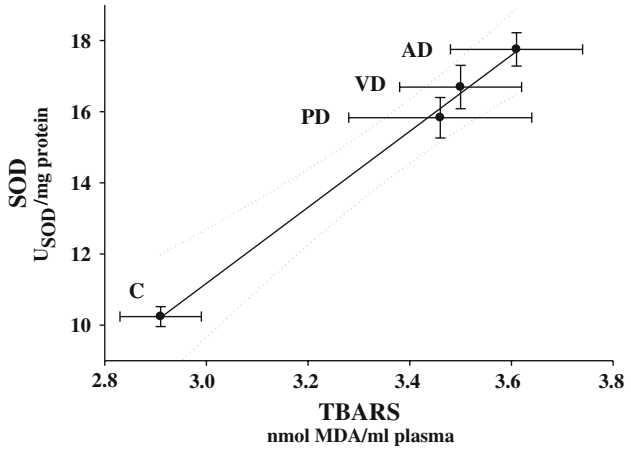

Fig. 2 Correlation between plasma TBARS and erythrocyte SOD values ($r = 0.998$). Data from Table 1. Statistics and abbreviations as in Fig. 1

superimposed diabetes, which is common in aged humans. Demographical and laboratory data are given in Table 2, and comprised 74 subjects and 222 observations.

In the presence of a simultaneous diabetic condition, plasmatic TBARS were increased in Alzheimer's disease (12%, $P < 0.05$) and in vascular dementia (5%, $P < 0.05$), whereas pure diabetic non-demented patients present a slightly decrease (−2%) non-significant; all the groups were compared against healthy controls. Plasma TRAP was significantly decreased in Alzheimer's disease (−30%, $P < 0.01$), in vascular dementia (−16%, $P < 0.05$), and in non-demented diabetic (−9%, $P < 0.05$). Erythrocyte's SOD was increased in Alzheimer's patients (13%, $P < 0.05$), in vascular dementia patients (36%, $P < 0.01$), and in non-demented diabetic (12%, $P < 0.05$). Also the differences in variables in the diabetic patients groups were compared with controls by means of the Bonferroni's statistic using setting $\alpha = 0.05$.

Again, the plasma levels of TBARS correlated, linearly and negatively, with plasma TRAP values ($r = -0.910$, $P < 0.001$, Fig. 3). The relationship between plasma TBARS and erythrocyte SOD values show a marked lack of linearity ($r = 0.274$, Fig. 4), as well as between plasma TRAP and erythrocyte SOD values ($r = -0.429$, not shown).

The comparison of Figs. 1, 2 and 3 with Fig. 4 shows that, when a diabetic condition is superimposed to the neurological diseases, the diseased groups are linearly correlated when the studied variables are plasma TBARS and plasma TRAP; when erythrocyte SOD is involved, the correlation is poor and their alignment is lost.

The associations between the measured variables were studied by means of Spearman's coefficient of correlation. A total of 115 pairs of variables were checked, and 15 of them are far away from the expected value (0 = no association). Numerical results are given in Table 3.

The larger number of patients/subjects from independent recruitment protocols, when analyzed simultaneously provides confirmation of the reproducibility of the results.

Table 2 Systemic oxidative stress in neurological diseases with superimposed diabetes. Demographical and laboratory data

Group/Age	TBARS	TRAP	SOD
Healthy controls (C)/73.9 ± 2.1 years	2.96 ± 0.11	452 ± 15	8.88 ± 0.42
$n_T = 19$ (7F/12M)	$n = 19$	$n = 19$	$n = 19$
Diabetes (DIAB)/66.9 ± 2.2 years	2.89 ± 0.13	412 ± 30*	9.97 ± 0.88*
$n_T = 18$ (8F/10M)	$n = 18$	$n = 18$	$n = 18$
Alzheimer's disease plus diabetes (AD + DIAB)/73.2 ± 1.5 years	3.30 ± 0.09*	317 ± 17**	10.05 ± 0.85*
$n_T = 19$ (12F/7M)	$n = 19$	$n = 19$	$n = 19$
Vascular dementia plus diabetes (VD + DIAB)/72.6 ± 1.4 years	3.09 ± 0.21*	378 ± 25*	12.07 ± 1.51**
$n_T = 18$ (5F/13M)	$n = 18$	$n = 18$	$n = 18$

Results are expressed as mean ± standard error

** $P < 0.01$, * $P < 0.05$ as compared with healthy controls

Units and abbreviations as in Table 1

Further analysis was conducted by one-way ANOVA's yielding the following F values for the differences in TBARS, TRAP, and SOD (222 observations, 74 for each variable), (v = degrees of freedom): TBARS ($F = 2.123$, $v = 3/70$, $P = 0.0518$); TRAP ($F = 6.724$, $v = 3/70$, $P = 0.000269$); SOD ($F = 2.499$, $v = 3/70$, $P = 0.0328$)

According to Bonferroni's method, a significance level of 0.05 is achieved when values are significant at the 0.00833 level; the corresponding critical value with the present design is $t_c = \pm 2.435$ for TBARS, TRAP, and SOD. The comparisons C vs. DIAB, C vs. AD + DIAB and C vs. VD + DIAB yielded the following t values: for TBARS ± 0.913 (not significant), ±5.513, and ±2.439; for TRAP ± 3.837, ±13.37, and ±7.098; for SOD ± 2.564, ±2.643, and ±6.997, respectively. It should be noted that mean age in the DIAB group was the smallest but that only the age comparison of C vs. DIAB resulted significant. Nevertheless, a large degree of overlap occurs between age distributions

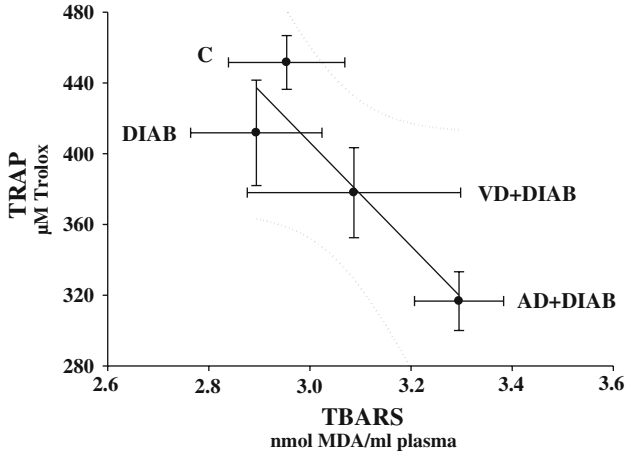

Fig. 3 Correlation between plasma TBARS and plasma TRAP values in diabetic patients ($r = -0.910$). Data from Table 2. Statistics as in Fig. 1. *C*, healthy controls; *DIAB*, diabetic patients; *AD + DIAB*, Alzheimer's disease patients with superimposed diabetes; *VD + DIAB*, vascular dementia patients with superimposed diabetes

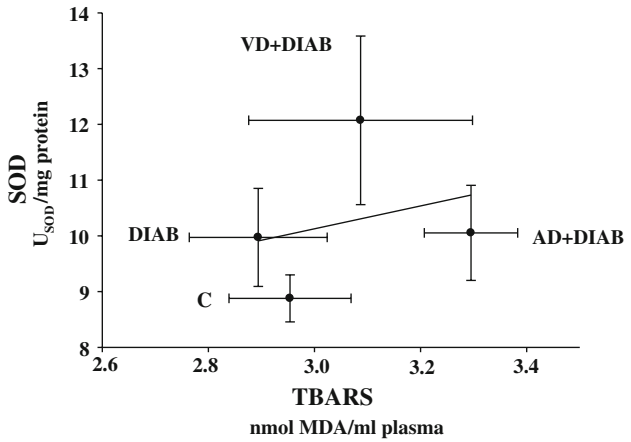

Fig. 4 Lack of relationship between plasma TBARS and erythrocyte SOD values in diabetic patients ($r = 0.274$). Data from Table 2. Statistics and abbreviations as in Fig. 3

Discussion

Systemic oxidative stress was found a common feature in Alzheimer and Parkinson's diseases and in vascular dementia. The concept of oxidative stress as coined by Sies implies an increase in the level of oxidants or a decrease in the level of antioxidants [45]. The concept has general validity and can be applied to in vitro biochemical system, isolated cells, tissue slices, whole organs or whole organisms.

The more common reactive species considered oxidants for cells and organs are: (1) the products of the partial reduction of oxygen, i.e., superoxide radical (O_2^{-}), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$), (2) free radicals ($R^{\bullet}$) and oxidizing free radicals ($ROO^{\bullet}$),

Table 3 Systemic oxidative stress in neurological diseases

Variables analyzed	Group	Sign of correlation (Spearman value)	Observations	Significance
TBARS/SOD	Global	Positive (0.302)	184	2.15×10^{-5}
TRAP/SOD	Global	Negative (−0.283)	227	1.05×10^{-5}
TBARS/SOD	C	Negative (−0.225)	33	9.8×10^{-2}
	VD	Negative (−0.318)	34	3.15×10^{-2}
	PD	Positive (0.389)	15	0.1
TBARS/CAT	AD	Positive (0.327)	18	0.1
	VD	Negative (−0.436)	15	0.1
TBARS/GPx	C	Negative (−0.389)	14	0.1
	AD	Positive (0.350)	18	0.1
	PD	Negative (−0.461)	15	5.0×10^{-2}
SOD/CAT	C	Negative (−0.329)	25	0.1
	AD	Positive (0.279)	33	5.4×10^{-2}
	VD	Negative (−0.344)	26	5×10^{-2}
SOD/Age	C	Negative (−0.426)	46	1.9×10^{-3}
	AD	Negative (−0.344)	65	2.8×10^{-3}

Associations between the measured variables

Abbreviations as in Table 1

intermediates of the chain reaction of lipid peroxidation, (3) hydroperoxides (ROOH) the *cuasi*-stable products of lipid peroxidation, (4) singlet oxygen ($^1O_2$), and (5) nitrogen containing reactive species, i.e., nitric oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^{-}$) and nitrogen dioxide (NO_2). The chemical species comprised in (1) to (4) are commonly named reactive oxygen species (ROS) and the ones in (5) are commonly termed reactive nitrogen species (RNS) [1].

Concerning intracellular antioxidants, the usually recognized and determined experimentally are: (1) the three antioxidant enzymes, i.e., superoxide dismutase, catalase and glutathione peroxidase, (2) reduced glutathione, and (3) the chain-marker antioxidant-vitamins, α-tocoferol, ascorbic acid, and β-carotene.

There is a current highly important consideration regarding the recognition of the situation of oxidative disbalance or oxidative stress in human subjects in physiological or pluripathological conditions or in aging. In the last years, it seems that a *consensus scholarum* emerged in which plasma levels of TBARS, TRAP, α-tocoferol, and ascorbic acid can be taken as indicators or as a systemic condition of oxidative disbalance. Additionally, there are many reports on erythrocyte's SOD, CAT, and GPx activities where they appear as markers of oxidative stress. Erythrocytes are representative cells because of their physiological role: oxygen free radicals and H_2O_2 are continuously generated by the auto-oxidation of hemoglobin [46].

Moreover, it has been reported that an antioxidant profile index considering some of the mentioned markers, including age, is able to discriminate between neurological pathologies with an 85% coincidence with clinical diagnosis: pathological groups from controls (93% success), AD (88.9%) from VD (73.3%), but not PD which overlaps with AD (60%) and VD (40%) [8]. This ability is supported by the different sign of the associations between pairs of variables in different clinical conditions, presented in Table 3. The significant associations (Spearman's correlation coefficient), between SOD/CAT, SOD/TBARS, TBARS/CAT, and TBARS/GPx have opposite signs in different pathologies, suggesting that, in spite of non-significant differences for CAT and GPx activities, they are related with the global phenomenon of oxidative stress. A marked difference between neurodegenerative and vascular diseases appears when AD and VD are compared. In the case of PD the differences are less defined and a clear cut separation between PD and VD cannot be established, probably because different metabolic free radical pathways are involved.

The patients of Alzheimer's disease (but not patients of other disease or in diabetes with concomitant Alzheimer's disease) showed SOD activities levels that were age-dependent and peaking at 70 years [5–9, 47, 48]. Two different linear regressions of the SOD activity as a function of age were found in AD patients below and above ~70 years (Fig. 5). In healthy humans, Junqueira et al. [36] reported that red blood cells SOD activity remains unchanged between 10 years age groups, but increases after 70 years; similar results were obtained for De la Torre et al. [49] that observed a significant increase of SOD activity in healthy Spanish people between 68 and 93 years.

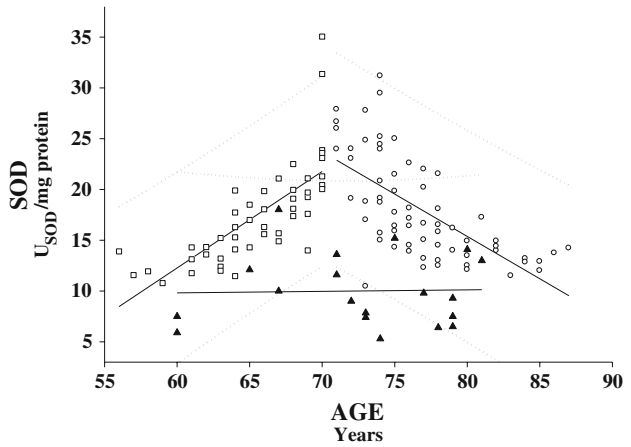

Fig. 5 SOD activity as a function of age in Alzheimer's patients. Individual values; □, pure Alzheimer's patients below 70 years ($n = 47$); ○, pure Alzheimer's patients above 70 years ($n = 65$); ▲, diabetic Alzheimer's patients ($n = 19$). *Solid lines* are the linear regressions ($r = 0.728$, 0.652, 0.026, respectively); *dotted lines* are the 99% prediction intervals; the lower limit of the Alzheimer's diabetic group is zero. Data are the raw values used to construct the means presented in Table 1

Type II diabetes, as a clinical condition superimposed to the described neurological disorders—a common feature in aged humans at neurological services—was found as a masking condition in terms of oxidative stress. The diagnostic or clinical control value of the antioxidant profile was hidden by the concomitant diabetic condition and/or the hypoglycemic drugs used [9]. The effect of the different hypoglycemic treatments was tested using a rank test; the results of the non-parametric technique show that, within the non-demented diabetic group patients treated with sulfonylurea showed a significant decrease in TBARS against other therapies. In the Alzheimer's diabetic group, patients undergoing treatment with sulfonylurea presented significantly lower levels of SOD; no differences were found within the vascular diabetic group, nor between the different sulfonylurea treatment employed (glibenclamide, glimepiride, and glicazide) [50]. No correlation was found in any group between age and the values of plasma TBARS and TRAP, other than the previously mentioned for SOD and age.

Considering the molecular rationale of oxidative stress, the significant results of the peripheral plasmatic variables (TBARS and TRAP) in the neurological diseased groups point to the existence of oxidative insult and systemic oxidative stress (SOS), alongside with the differences indicated by markers representing conditions that change in a few days period. Moreover, the erythrocyte markers (SOD, CAT, and GPx) show significant changes in SOD but slight and non-significant for CAT and GPx; the oxidative insult and systemic oxidative stress associated with the diseases is confirmed through a marker representing the homeostatic long term response of the bone marrow in the order of 60–80 days.

The existence of a systemic oxidative stress is reinforced when both plasmatic and erythrocyte markers are considered, which represent the oxidative disorders in a wide temporal range resulting from the excess of hydroperoxides in chronic conditions. However, definitive evidence for the association between neuronal and peripheral markers of SOS is still unclear [51]. In spite of these shortcomings, the present findings suggest that free radical-mediated neuronal damage may be detected by the used peripheral markers.

It is also interesting to note the coincidence resulting from the linear ordering of the responses in relation with the complexity of the pathologies, accordingly with the simple Sies scale model. *The steady-state formation of prooxidants in cells and organs is balanced by a similar rate of their consumption by antioxidants that are enzymatic and/or non-enzymatic. Oxidative stress results from*

imbalance in this prooxidant/antioxidant equilibrium in favor of the prooxidants [11].

From the viewpoint of the molecular rationale of the oxidative stress, the results of the diabetic groups are more difficult to explain. The observed changes in the peripheral markers (TBARS and TRAP) in the Alzheimer's plus diabetes and vascular dementia plus diabetes groups with respect to controls are significant, but within a general phenomenon of a marked numerical attenuation in the whole diabetic population. These experimental results also show a paradoxical phenomenon when diabetes is superimposed to dementia [9, 48]. Such attenuation might be attributed to: (a) the well known decreasing action of sulfonylurea on TBARS and SOD; (b) a somewhat "protecting effect" of diabetes as a buffer of the systemic oxidative stress due to failure in the response of the bone marrow as a consequence of diabetes complications and tissue damage; (c) a protective action of insulin against oxidative stress, reported by De Felice et al. [52], among other effects related with specific damage in AD disease. Thus, the increased levels of insulin could explain the observed attenuation in diabetic patients. Perhaps, the most surprising "protective effect" of diabetes was described in association with metastasis in patients with malignant tumors, such as in lung and prostate cancer [53–56].

While SOD is significantly increased in diabetic and diabetic-demented groups against controls the linearity of the responses disappears (Fig. 4). The non-aligned point is SOD in the Alzheimer's with concomitant diabetes group, which should be some four to five units (130–160 pmol of SOD) larger to reproduce the linearity observed in non-diabetic patients (Figs. 2, 4). It seems that in the Alzheimer's diabetic patients the adaptive response of the bone marrow fails, not producing enough SOD to reach the high values needed to achieve the "balance" between the variables underlying the observed linearity. This experimental result should be considered consistent with the greater deterioration associated with the Alzheimer's disease per se.

It is also to be noted that the degree of oxidative stress does not predict the clinical condition in Alzheimer's patients. The molecular basis for this fact is unclear but might be related with the importance of steady-state kinetics in the induction of oxidative stress by oxidative damage [57]. Steady-state concentrations of active species of oxidants are, for instance, superoxide radical (O_2^-): 10^{-10}M, hydrogen peroxide (H_2O_2): 5×10^{-9}M, hydroxyl radical ($HO^\bullet$): 10^{-18}M, nitric oxide radical ($NO^\bullet$): 10^{-9}M, peroxynitrite ($ONOO^-$): 10^{-8}M [2]. The superoxide radical is a precursor of most other ROS but it is not a strong oxidant, being involved in the propagation of oxidative chain reactions, reacting with other species including one of the RNS, nitric oxide which produces peroxynitrite, also a very powerful oxidant.

Variations in the levels of the steady-state concentration of oxidants/prooxidants result in chain reactions that indiscriminately target proteins, lipids, polysaccharides, and DNA. The balance between antioxidant defenses and ROS production depends on genetic factors, aging, and inhibition of electron flow or exposure to xenobiotics. Both genetic factors and aging may cause an increased mitochondrial steady-state concentration of ROS; the mitochondrial respiratory chain constitutes its main intracellular source. The levels are maintained at non-toxic concentrations by several antioxidant defenses and repair enzymes [58]. An imbalance appears as a common denominator in various pathological processes in which the resulting oxidative insult causes tissue damage and, eventually, cells death; remarkably, this is the case of the diseases studied here. In view of the depressed SOD values in AD + DIAB patients in relation with AD, it is possible to hypothesize that in the presence of superimposed diabetes the steady-state concentrations of the superoxide free radical species are lowered.

There is also basic and clinical research in antioxidant defenses and in SOD induction, both related with age. With regard to the role of antioxidant defenses, studied in healthy subjects, an age-related significant increase in α-tocopherol and β-carotene concentrations was reported for the older age groups against the 20–30 years old up to the 50–60 years group. From that age onwards these antioxidant substances decrease significantly in spite that the daily intake (natural or dietary supplemented) remains unchanged. In turn, the ascorbic acid concentration does not show variations between age groups [36]. With respect to SOD it was found that its induction is highly age-dependent and that, in humans is no longer inducible beyond ca. 70 years old [59–61].

It should also be considered that other biochemical variables proposed as markers of dementias such as the methionine cycle (i.e.: plasmatic total homocysteine, vitamin B12 and folate) and plasmatic insulin, glucose and glycated hemoglobin, also show significant differences between dementias and dementias with a superimposed diabetic condition. In the methionine cycle, v.g., diabetes affects plasmatic levels of total homocysteine, vitamin B12 and folate, which are changed with opposite sign if diabetes is present [62]. Moreover, the correlation's insulin/glucose and insulin/glycohemoglobin are positive in demented groups while the correlation is negative in controls and non-demented diabetic patients [63]. These results are well in line with the reported protective effect against oxidative stress and synapses protection [52].

The present results show linearity between pairs of oxidative stress variables and the ordering of the patients' conditions along the different regression lines pointing to the existence of an overall balance between oxidative

insult, damage and protection as proposed in the hypothesis.

Conclusions

Finally it can be concluded that, among other biochemical peripheral markers for dementias, oxidative insult and a significantly systemic oxidative stress are associated with neurological diseases of aging and that the involvement of enhanced but different free radical metabolic pathways are implicated as characteristic of each disease.

Acknowledgments This study has been partially supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Fundación Argentina Contra las Enfermedades Neurológicas del Envejecimiento (FACENE) of Argentina.

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