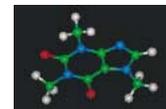


Lymphocyte Oxidative DNA Damage and Plasma Antioxidants in Alzheimer Disease



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Context: A large body of experimental evidence suggests that in Alzheimer disease (AD) pathogenesis an important role is played by oxidative stress, but there is still a lack of data on in vivo markers of free radical-induced damage.

Objectives: To evaluate levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative damage to DNA, in peripheral lymphocytes; to measure plasma concentrations of several nonenzymatic antioxidants; and to assess the relationships between any observed changes in lymphocyte DNA 8-OHdG content and plasma antioxidant levels in patients with AD and healthy aged control subjects.

Subjects: Forty elderly outpatients with AD and 39 healthy age- and sex-matched controls were studied.

Main Outcome Measures: The level of 8-OHdG was determined in DNA extracted from lymphocytes and plasma levels of vitamin C, vitamin A, vitamin E, and ca-

rotenoids (zeaxanthin, β -cryptoxanthin, lycopene, lutein, and α - and β -carotene) were measured by high-performance liquid chromatography.

Results: Lymphocyte DNA 8-OHdG content was significantly higher and plasma levels of antioxidants (with the exception of lutein) were significantly lower in patients with AD compared with controls. In patients with AD, a significant inverse relationship between lymphocyte DNA 8-OHdG content and plasma levels of lycopene, lutein, α -carotene, and β -carotene, respectively, was observed.

Conclusions: Markers of oxidative damage are increased in AD and correlate with decreased levels of plasma antioxidants. These findings suggest that lymphocyte DNA 8-OHdG content in patients with AD reflects a condition of increased oxidative stress related to a poor antioxidant status.

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A LARGE body of research suggests that oxidative stress, a condition representing an imbalance between oxidants and antioxidants in favor of the oxidants,¹ plays an important role in the pathogenesis of Alzheimer disease (AD).² The brain is particularly susceptible to the attack of free radicals caused by its low content of antioxidants, by the considerable content of polyunsaturated fatty acid side chains of the neuronal membrane lipids, and by its high oxygen consumption rate.^{2,3} Furthermore, aging—a major risk factor for AD—is known to be associated with increased free radical production, and markers of oxidative DNA damage have been found to be increased in cerebral tissue of healthy aged subjects⁴ and even more so in patients with AD.⁵ The initial idea of a link between increased free radical production and AD⁶ is now supported by stud-

ies showing a potential vicious circle between free radical-induced β -amyloid formation and β -amyloid-related oxidative stress.⁷

Although the brain is the most affected organ in AD, several studies showed structural or functional alterations in peripheral tissues. In patients with AD, alterations of β -amyloid metabolism⁸ or of glutamate uptake⁹ were detected in platelets and increased isoprostane levels in urine, blood, and cerebrospinal fluid.¹⁰ We also found a higher level of oxidative damage to DNA in lymphocytes.¹¹ Recently, a reduced expression of heme-oxygenase messenger RNA in lymphocytes was reported.¹²

The aims of the present study were (1) to evaluate levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG)—a well-established marker of oxidative stress in DNA—in peripheral lymphocytes; (2) to measure plasma concentrations of several nonenzymatic

SUBJECTS AND METHODS

Forty elderly outpatients (20 women and 20 men; mean [SD] age, 75.9 [5.4] years) with mild to moderate AD (mean [SD] Mini-Mental State Examination score, 17.3 [2.1] points; range, 14-23 points) diagnosed on the basis of DSM-IV-R¹³ and of NINCDS-ADRDA (National Institutes of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association) criteria¹⁴ were included in this study. Patients were compared with 39 healthy aged subjects (20 women and 19 men; mean [SD] age, 74.8 [6.3] years) with a Mini-Mental State Examination score ranging between 27 and 30 points. Patients and controls with a history of having a smoking habit and/or alcohol abuse, major organ failure, dyslipidemia, or alteration of protein metabolism, as well as those taking iron or antioxidant supplements were excluded. The Mini Nutritional Assessment¹⁵ was administered to all participants. Body mass index (calculated as weight in kilograms divided by height in meters, squared), plasma albumin, and transferrin were evaluated in all subjects with the aim to select well-nourished persons. A semiquantitative food frequency questionnaire was used to evaluate the dietary habits of patients and controls.

After giving informed consent, patients and controls underwent a 20-mL blood sample withdrawal for measurement of 8-OHdG in lymphocytes, and of vitamin C (ascorbic acid), vitamin A (retinol), vitamin E (α -tocopherol), and carotenoids (namely, zeaxanthin, β -cryptoxanthin, lycopene, lutein, α -carotene, and β -carotene) in plasma.

Lymphocytes were separated, DNA was extracted, and 8-OHdG was assayed as previously described.¹¹ Briefly,

freshly obtained blood was layered on Lymphoprep (Gibco BRL, Bethesda, Md), centrifuged and washed, and the pellet was stored at -80°C until analysis. DNA was extracted from the cells with DNAzol (Gibco BRL), resuspended with 10mM of Tris hydrochloride and 1 mM of EDTA, and enzymatically hydrolyzed. Both 8-OHdG and deoxyguanosine (dG) were measured by high-performance liquid chromatography with electrochemical and UV detection, respectively. Results are expressed as 8OHdG molecules per 10^5 dG molecule (8OHdG-dG ratio).

Vitamin C was detected by high-performance liquid chromatography with electrochemical detection according to Kutnink et al¹⁶ with a Supelco C18 column (250 mm \times 4.6 inner diameter) and a Supelco C18 guard column (20 mm \times 4.6 inner diameter). Vitamins A and E were measured, after extraction with ethanol and hexane, by high-performance liquid chromatography with UV detection at 280 nm¹⁷ with a Waters Symmetry C8 column (150 mm \times 4.6 inner diameter). Carotenoids were detected after extraction with hexane-dichloromethane (5:1) by high-performance liquid chromatography at 450 nm using a Supelco C18 column (250 mm \times 4.6 inner diameter).¹⁸

Statistical analysis was performed with the program StatView 4.5 (Abacus Concept; SAS Institute, Cary, NC). All data are presented as mean (SD). Nonparametric Mann-Whitney test was used for comparisons between groups and χ^2 analysis for frequency evaluation. Spearman rank test was performed to evaluate the relationship between variables. Since considering the ratio between plasma vitamin E/vitamin A/carotenoids/and total cholesterol level did not alter results, data are expressed as total plasma levels of these compounds.

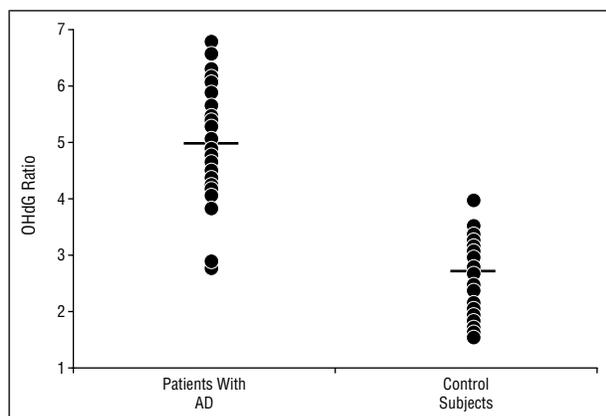


Figure 1. Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), expressed as 8OHdG molecules per 10^5 dG molecule ratio (OHdG), in the lymphocytes of patients with Alzheimer disease (AD) and control subjects.

antioxidants; and (3) to assess the relationships between any observed changes in lymphocyte DNA 8-OHdG content and plasma antioxidant levels in elderly subjects and patients with late-onset AD.

RESULTS

As shown in **Figure 1**, DNA extracted from lymphocytes of patients with AD contained significantly higher amounts of 8-OHdG (4.79 [1.08] 8-OHdG/ 10^5 dG; range, 2.5-6.6

Plasma Concentrations of Antioxidants in Patients With Alzheimer Disease (AD) and in Healthy Aged Control Subjects*

| Compound | Patients With AD, $\mu\text{mol/L}$ (n = 40) | Control Subjects, $\mu\text{mol/L}$ (n = 39) |
|-----------------------------------|----------------------------------------------|----------------------------------------------|
| Vitamin C (ascorbic acid) | 32.28 (10.8) | 56.74 (15.9) |
| Vitamin A (retinol) | 3.52 (0.76) | 4.87 (0.84) |
| Vitamin E (α -tocopherol) | 37.94 (6.80) | 47.70 (6.28) |
| Zeaxanthin | 0.06 (0.03) | 0.11 (0.04) |
| β -Cryptoxanthin | 0.03 (0.02) | 0.30 (0.19) |
| Lycopene | 0.29 (0.13) | 0.72 (0.26) |
| Lutein | 0.35 (0.18) | 0.38 (0.17) |
| α -Carotene | 0.04 (0.02) | 0.06 (0.03) |
| β -Carotene | 0.18 (0.06) | 0.52 (0.23) |

*Data are given as mean (SD). All of the P values were $<.001$, except lutein, which was not statistically significant.

8-OHdG/ 10^5 dG) compared with controls (2.72 [0.78] 8-OHdG/ 10^5 dG; range, 1.3-4.1 8-OHdG/ 10^5 dG) ($P<.001$). Plasma levels of vitamin C, vitamin A, vitamin E, and the carotenoids are listed in the **Table**. Mean plasma antioxidant levels were lower in patients compared with controls. Differences reached statistical significance for all compounds measured, with the exception of lutein.

In patients with AD, we found a significant inverse relationship between the content of 8-OHdG in lympho-

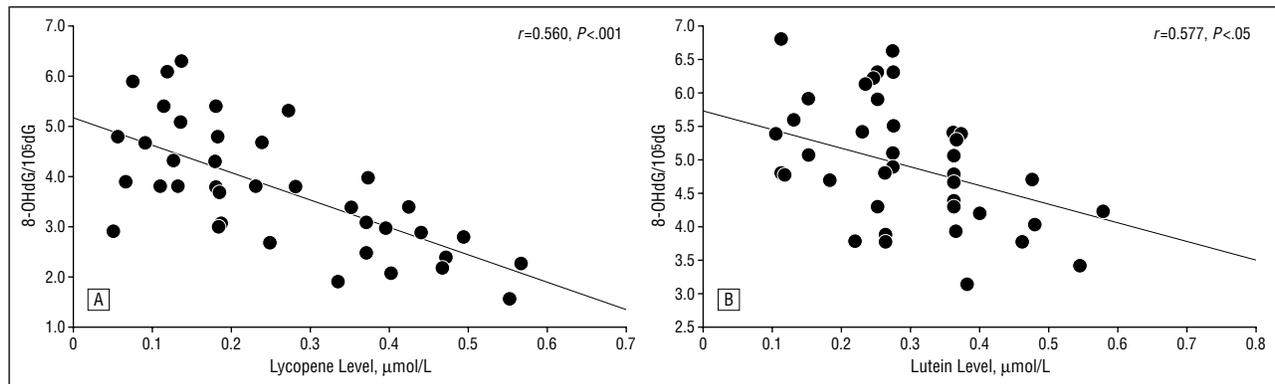


Figure 2. Linear regression analysis between lymphocyte 8-hydroxy-2'-deoxyguanosine (8-OHdG) and, respectively, lycopene (A) and lutein (B) in patients with Alzheimer disease (AD).

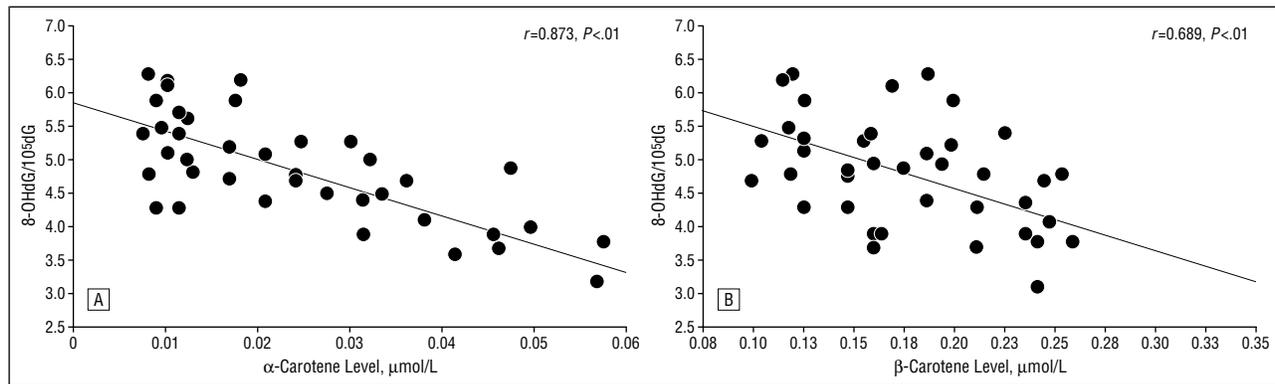


Figure 3. Linear regression analysis between lymphocyte 8-hydroxy-2'-deoxyguanosine (8-OHdG) and, respectively, α-carotene (A) and β-carotene (B) in patients with Alzheimer disease (AD).

cyte DNA and plasma levels of lycopene ($r = 0.560$, $P < .001$) (**Figure 2A**), lutein ($r = 0.577$, $P < .05$) (**Figure 2B**), α-carotene ($r = 0.873$, $P < .01$) (**Figure 3A**), and β-carotene ($r = 0.689$, $P < .01$) (**Figure 3B**). No correlation was found between the content in 8-OHdG of lymphocyte DNA and antioxidants in controls.

No significant differences were found between the patients with AD and controls regarding body mass index, plasma albumin and transferrin levels, Mini Nutritional Assessment scores, and dietary habits (particularly with respect to frequency of fruit and vegetable intake).

COMMENT

Oxidative damage may play an important role in the pathogenesis of several neurodegenerative diseases, and growing evidence points to the involvement of free radicals in mediating neuronal death in these illnesses. Oxygen free radicals, which are mainly products of mitochondrial activity, react with all key cellular components. As a consequence of this, damage to purines and pyrimidines in DNA yielding oxidized bases is produced. Among oxidized bases, 8-OHdG is the most abundant, and it can be measured in both tissue extracts and cells. It has, therefore, been proposed as a marker of oxidative damage both in physiological and pathological conditions.

Our prior work and others showed increased amounts of 8-OHdG in mitochondrial and nuclear DNA from

AD-affected brains compared with controls.^{5,19} Increased levels of 8-hydroxyguanine were also found in intact DNA isolated from ventricular cerebrospinal fluid in autoptic samples from subjects with AD.²⁰ Postmortem studies, however, are not easy to perform, and the feasibility of detecting 8-OHdG in peripheral cells, such as lymphocytes, may be useful in evaluating conditions of oxidative stress.

In the present study, we detected significantly increased levels of 8-OHdG in lymphocyte DNA from patients with AD compared with controls. We found that the increased content of 8-OHdG is significantly correlated with a decreased content of plasma carotenoids in patients with AD. Although it has been proposed that carotenoids protect against degenerative conditions, such as cancer and coronary heart disease, direct evidence of their *in vivo* antioxidant activity is limited.^{21,22} Furthermore, the role of antioxidants in preventing neurodegenerative disorders, and in particular AD, is scanty.

Measurement of peripheral antioxidants is considered an appropriate way of looking at oxidative stress in various disease states in humans.²³ In our study, all measured plasma antioxidants were significantly lower in patients with AD than in healthy controls. A potential explanation of the low levels of antioxidants in patients with AD is that these patients are malnourished. Our patients, however, did not differ from controls with respect to dietary habits, Mini Nutritional Assessment score, body mass index, or plasma albumin and transferrin levels. Low plasma levels of vitamin C, moreover, were

found in AD despite an adequate diet.²⁴ Another possible explanation for low levels of plasma antioxidants in AD is that they might be consumed because of a disease-related higher rate of free radical production. In AD, decreased cytochrome *c* oxidase activity has been shown both in cerebral tissue²⁵ and peripheral cells,²⁶ as well as a pro-oxidant activity that has been attributed to β -amyloid.²⁷ Some epidemiological studies showed that low plasma levels of antioxidant vitamins are a risk factor for cognitive impairment,²⁸⁻³¹ and that oxidative stress markers are associated with cognitive decline.³² We also found high levels of vitamin A and vitamin E in plasma samples of mentally healthy centenarians,³³ suggesting a protective role of these nutrients in the oldest-old. Antioxidant vitamin supplementation seems to decrease the risk of cognitive impairment, as suggested by studies in which ascorbic acid and α -tocopherol administration lowered the risk of suffering from AD³⁴ or, more generally, from dementia.³⁵ Therapeutic use of antioxidants (α -tocopherol and/or selegiline) has been also proposed to slow the course of AD.³⁶ Although there are only few studies on the relationship between plasma and brain concentration of antioxidants, it has been demonstrated that supplementation or depletion of vitamin E results in marked changes in vitamin E levels in the rat brain^{37,38} and α -tocopherol and ascorbic acid supplementation has been shown to increase the concentrations of both vitamins not only in plasma, but also in cerebrospinal fluid.³⁹ The relationship between markers of oxidative stress in the brain and at the peripheral level has been studied in a transgenic mouse model of AD amyloidosis by Praticó et al⁴⁰ who measured isoprostanes-specific and -sensitive markers of *in vivo* lipid peroxidation in cerebral tissue, plasma, and urine. In these animals, brain, plasma, and urine isoprostanes levels were higher compared with control mice and also brain levels were positively correlated to amyloid deposition. Other data have also shown that oxidative stress increases intracellular β -amyloid.^{41,42}

Several researchers show that biomarkers of oxidative stress are present in AD peripheral tissues.⁴³ In particular, increased concentrations of isoprostanes were detected in urine, plasma, and cerebrospinal fluid samples of patients with AD. A direct correlation was found between plasma and cerebrospinal fluid levels, suggesting that plasma levels may reflect brain oxidative stress.¹⁰ It is possible that AD-affected lymphocytes are genetically prone to oxidative stress. It has been recently shown that peripheral lymphocytes from presenilin-1 transgenic mice have an enhanced vulnerability to cell death, which was associated with an increased production of reactive oxygen species and altered calcium regulation,⁴⁴ linking a genetic cause of AD to a condition of oxidative stress in peripheral cells. It was also found that glutathione S-transferase polymorphisms influence the level of oxidative DNA damage in lymphocytes. The damage was more marked if subjects had poor antioxidant status.⁴⁵ So it is conceivable that the oxidative DNA damage, such as lymphocyte 8-OHdG, could result from co-occurrence of pro-oxidant genetic and environmental factors.

In the present study, we observed an inverse trend between plasma antioxidants and the content of 8-OHdG

in lymphocyte DNA of patients with AD. There was a strong, significant relationship for lycopene, lutein, and α - and β -carotenes. A significant negative correlation between concentrations of serum carotenoids and oxidized pyrimidines was found in lymphocytes of healthy humans,⁴⁶ suggesting that some nutritional factors may protect from oxidative DNA damage (as assessed by the presence of DNA strand breaks measured with the comet assay). In another study, Lenton et al⁴⁷ showed that naturally occurring levels of intracellular antioxidants (glutathione and vitamin C) were negatively correlated with oxidative damage in human lymphocytes, as assessed by levels of 5-hydroxy-2'-deoxycytidine and of 8-oxo-dG. In particular, the strongest inverse relationship was found between glutathione and 8-oxo-dG, and, on the basis of these results, the authors suggested that intracellular glutathione and ascorbate protect human lymphocytes against oxidative DNA damage.⁴⁷ Several studies have shown that dietary supplements including ascorbic acid, α -tocopherol, and β -carotene protect lymphocyte DNA against oxidative damage.⁴⁸ Flavonoids,⁴⁹ coenzyme Q10,⁵⁰ and lycopene contained in tomatoes⁵¹ have been shown to exert protective effects against oxidative DNA damage, either by decreasing DNA strand breaks⁵² or by increasing DNA resistance to hydrogen peroxide (H_2O_2)-induced oxidation.^{50,51} High levels of the xanthophylls lutein and β -cryptoxanthin are related to low levels of lymphocyte 8-OHdG and of urinary 8-epiprostaglandin $F_{2\alpha}$. Supplementation with xanthophylls and lycopene further reduces the amount of these biochemical markers of oxidation.⁵² β -Carotene supplementation also reduced oxidative damage in DNA of lymphocytes treated with H_2O_2 .⁵³ Furthermore, it was recently shown that exposure of mouse monocytes to oxidized low-density lipoproteins results in an accumulation of 8-OHdG in DNA and in a down-regulation of base excision repair activity. Treatment with antioxidants (in this case ascorbate and α -tocopherol) reversed this situation, showing a linkage between lipid and DNA oxidation and between lipid oxidation.⁵⁴

CONCLUSIONS

This preliminary study suggests that lymphocyte DNA 8-OHdG levels are increased in AD and correlate with decreased levels of plasma antioxidants. These findings suggest that lymphocyte 8-OHdG levels in patients with AD reflects a condition of increased oxidative stress related to a poor antioxidant status. It will be important to replicate the findings of this study in other populations and at different severity stages of dementia.

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(Dr Cherubini); obtained funding (Drs Mecocci and Senin); administrative, technical, and material support (Drs Ingegneri and Mattioli); study supervision (Drs Catani and Beal).

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