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Serum Levels of Antioxidant Vitamins and Lipid Peroxidation in Multiple Sclerosis

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We determined serum levels of ascorbic acid, beta-carotene, retinol and alpha tocopherol and lipid peroxidation (as estimated by thiobarbituric acid reacting substances (TBARS) generation) in 24 multiple sclerosis (MS) patients and 24 healthy sex- and age-matched person as control. The levels of four antioxidant vitamins were significantly lower in MS patients compared to controls ($p < 0.05$). TBARS levels were significantly higher in the patients of MS compared to the controls ($p = 0.001$). In MS patients, the levels of beta-carotene, alpha tocopherol and ascorbic acid correlated significantly with each other ($r^2 = 0.689 - 0.779$). It appeared that there was inverse correlation between the serum levels of ascorbic acid or beta-carotene, but not of alpha tocopherol or retinol, and TBARS levels in MS. The present study indicates that antioxidant vitamins (alpha tocopherol, beta-carotene, retinol and ascorbic acid) are decreased in sera of MS patients during an attack, and that this decrease may well be dependent on the increased oxidative burden as reflected by lipid peroxidation products. The role of antioxidant vitamin supplementation in prevention and/or treatment of MS remains to be explored.

Keywords: Alpha tocopherol; Ascorbic acid; Beta-carotene; Multiple sclerosis; Retinol; Thiobarbituric acid reactive substances

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of unknown origin (Karg *et al.*, 1999; Noseworthy *et al.*, 2000). Genetic susceptibility and environmental influences are the risk factors involved in MS. Relapsing–remitting MS—the type present in 80% of patients—typically begins in the second or third decade of life and has a female predominance of approximately 2:1. Persistent signs of central nervous system dysfunction may develop after a relapse, and the disease may progress between relapses (secondary progressive MS). Twenty percent of the affected patients have primary progressive MS, which is characterized by a gradually progressive clinical course and a similar incidence among men and women (Noseworthy *et al.*, 2000). The risk of developing MS in the general population is approximately 0.2%. However, the risk to members of families who have a father, mother, sister, or brother with MS ranges from 1.0 to 6.0%. In general, rates increase with latitude, although there are some noteworthy exceptions to this geographic gradient (McMichael and Hall, 2001).

Perturbation of the cellular oxidant/antioxidant balance has been suggested to be involved in the neuoropathogenesis of several disease states, including stroke, MS, Parkinson disease, Alzheimer's disease as well as "normal" physiological aging (Calabrese *et al.*, 2000; Noseworthy *et al.*, 2000). It has been suggested that there is a possible role of free radicals, including nitric oxide, in the pathogenesis of MS (Hunter *et al.*, 1985; Bö *et al.*, 1994; Calabrese *et al.*, 2000). Increased lipid peroxidation has been observed both in the cerebrospinal fluid and in the blood of MS patients. In addition to the antioxidant enzymes, namely catalase, superoxide dismutase, glutathione peroxidase and glucose-6-phosphate dehydrogenase, the blood and some other tissues

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contain nonenzymatic antioxidants, namely alpha tocopherol, beta-carotene, retinol and ascorbic acid, among others. The data regarding the relationship between antioxidant vitamins and MS is limited. Warren (1982) found that low dietary intake of vitamin A, vitamin E and selenium but not of beta-carotene or ascorbic acid, are associated to be a risk factor for the onset of the disease. Further, two studies have found that the plasma levels of alpha tocopherol, beta-carotene, retinol and ascorbic acid are similar in MS patients and in controls (Wikström *et al.*, 1976; Wong *et al.*, 1993). The data, however, are scarce and conflicting. The aim of this study was, therefore, to investigate the possible changes in antioxidant vitamin levels and their relationship to the oxidative stress produced by MS. For this purpose, we measured the serum levels of antioxidant vitamins, alpha tocopherol, beta-carotene, retinol and ascorbic acid and of thiobarbituric acid reactive substances (TBARS) in a group of secondary progressive MS patients who first experience exacerbation, and compared them to levels obtained from healthy controls.

MATERIALS AND METHODS

Patients and Controls

Twenty-four patients (35.9 ± 7.3 years) with secondary progressive MS were included in the present study. The diagnosis of definite MS was confirmed according to the clinical and laboratory diagnostic criteria of the Poser Committee (1983), and 24 healthy sex- and age-matched person (36.8 ± 4.1 years) without any diseases, who also had normal physical examination findings, served as control group. Secondary progressive MS patients who first experience exacerbations were recruited from outpatients making the first visit to the departments of neurology or the physical therapy and rheumatology of two hospitals based in the region of Ankara. Neurologic status and progression of disease were evaluated by the Kurtzke disability status scoring. The patients and healthy controls had received neither steroid therapy nor vitamin supplementation, and none were smokers. Informed consent according to the declaration of Helsinki was obtained from each subject at the time of recruitment to the study, which was approved by the Research Ethical Committee of the Hospitals, Ankara.

Sample Collection and Preservation

From each patient and healthy age- and sex-matched person, 10 ml of blood from the antecubital vein were taken. Blood was always collected while fasting during the morning hours, and centrifuged at 4°C for 10 min at 2500 rpm to obtain serum. The serum samples were freshly frozen under nitrogen and stored at −70°C until analysis. Samples were not stored longer than six months. The samples were thawed at room temperature only once at the time of assay.

Determination of Vitamins and Lipid Peroxide Levels

Serum alpha tocopherol concentration was measured by using the method described by Desai (1984). In short, the mixture containing 0.5 ml volume of serum, 0.5 ml of ethanol and 0.25 ml of 25% ascorbic acid was preincubated at 70°C for 5 min. Following incubation, 0.3 ml of saturated potassium hydroxide was added and the mixture was further incubated at 70°C for 30 min. Tubes were cooled immediately in an ice bath. A 4.0 ml of hexane was added into tubes. Duplicate samples were extracted in glass-stoppered centrifuge tubes, followed by vigorous vortex mixing for 1 min and centrifugation at 1500 rpm for 5–10 min. After separation of phases, hexane extracts were used to estimate the concentration of the vitamin level on a spectrofluorometer (Jasco FP-750). Excitation and emission wavelengths were 286 and 330 nm, respectively. Alpha tocopherol concentration was obtained directly from the standard curve. The intra- and inter-assay of coefficient variation for the measurement were 1.9 and 3.1%, respectively. Beta-carotene and retinol were determined by using the method of Neeld and Pearson (1963). Briefly, duplicate serum samples (0.5 ml) were mixed with 1.0 ml of cold ethanol and then extracted with 2.0 ml of hexane, followed by being vortex mixed for 2 min. The mixture was centrifuged at 800*g* for 10 min. Beta-carotene was determined spectrophotometrically at 450 nm (Unicam 8700 series). Retinol was extracted into petroleum ether in the presence of ethanol and was reacted with trifluoracetic acid, which leads to the dehydrated anhydrovitamin, which was then measured spectrophotometrically at 620 nm (Unicam 8700 series). Both beta-carotene and retinol concentrations were obtained directly from their standard curves. Intra- and inter-assays of coefficient variation in beta-carotene measurements were calculated as 1.6 and 1.7%, and retinol measurements were 2.6 and 3.1, respectively. Serum ascorbic acid was measured spectrophotometrically at 520 nm after its reaction with 2,6-dichlorophenolindophenol (Kalaycı *et al.*, 2000). The intra- and inter-assay variations for the vitamin C assay in this study were 2.1 and 3.2%, respectively.

Serum lipid peroxide levels were determined measuring thiobarbituric acid (TBA) reactivity as described by Wade and van Rij (1988). Briefly, 200 μl of trichloroacetic acid (TCA) (25 g TCA in 10 ml distilled water) was added to 1 ml of serum. The

TABLE I Characteristics and some analytical data of patients with MS and control groups

Parameters	MS patients ($n = 24$)	Control ($n = 24$)
Characteristics		
Age (years)	35.9 ± 7.3†	36.8 ± 4.1
Gender male/female (n)	16/8	16/8
Analytical data		
Retinol (μmol/l) (Ranges)	2.07 ± 0.21* (1.39–2.48)	2.53 ± 0.26 (1.67–3.29)
Beta carotene (μmol/l) (Ranges)	0.41 ± 0.13* (0.12–0.72)	0.63 ± 0.08 (0.34–0.87)
Ascorbic acid (μmol/l) (Ranges)	31.19 ± 3.87* (22.30–40.28)	49.05 ± 3.69 (35.94–58.32)
Alpha tocopherol (μmol/l) (Ranges)	22.05 ± 2.05* (16.74–25.31)	26.20 ± 1.99 (21.44–33.28)
TBARS (μmol/l) (Ranges)	3.93 ± 0.12* (3.63–4.23)	1.89 ± 0.03 (1.46–2.20)

*$p < 0.05$ MS patients vs. the controls. † $\bar{X}$ ± SD.

mixture was centrifuged at 1000g for 10 min, and the precipitate was reacted with 1 ml of 0.67% TBA (w/v). The samples were heated at 100°C for 30 min. After centrifugation, the absorption of malondialdehyde (MDA)–TBA chromogen was measured at 532 nm. Tetramethoxy propane was used as MDA standard. TBA reactivity was calculated as micromole MDA per liter (μmol/l). Intra- and inter-assay of coefficient variation averaged 5.1 and 6.2%, respectively.

Statistical Analysis

All statistical analyses were conducted using "SPSS 10.0 for Windows" (SPSS Inc., USA) statistical program. Mann–Whitney U test was used, and comparison of MS patients and controls. Correlations between parameters were studied using Person's test. Values are expressed as mean ± standard deviation (SD). A significance level (p value) of 5% was used unless stated otherwise.

RESULTS

Mean ages of the patients and healthy controls were 35.9 ± 7.3 and 36.8 ± 4.1 years, respectively. No significant differences appeared between the groups. The results are shown in Table I.

Serum levels of ascorbic acid, alpha tocopherol, retinol and beta-carotene were found to be lower in MS patients during exacerbation than in the healthy age- and sex-matched control group ($p < 0.05$). MS patients had significantly higher serum TBARS levels (3.94 ± 0.12 μmol/l) compared to the control group (1.81 ± 0.15 μmol/l) ($p = 0.001$).The possibility of gender based differences in the antioxidant vitamins and a marker of lipid peroxidation products, TBARS in MS patients was also examined by Wilcoxon signed rank test. It appeared that there were no differences in the levels of antioxidant vitamins and TBARS of the patients with MS when the data was dissected by gender (data not shown).

Interestingly, in MS patients the levels of beta-carotene, alpha tocopherol and ascorbic acid correlated significantly with each other, with r^2 values ranging between 0.689 and 0.779 (Fig. 1a–e).

It appeared that there was inverse correlation between the serum levels of TBARS and the antioxidant vitamins namely ascorbic acid or beta-carotene in MS patients (Fig. 2a and b). No correlation was observed between the serum levels of TBARS and alpha tocopherol or retinol (data not shown).

DISCUSSION

In the present study, the results confirm previous observations that there is an oxidative stress in patients with MS and further extend the concept that this oxidative stress is associated with significantly decreased levels of all four antioxidant vitamins (ascorbic acid, alpha tocopherol, retinol and beta-carotene), and increased levels of TBARS.

Endogenous antioxidant defenses in body were not completely effective for neutralization of oxidative stress (Gey, 1998). Dietary antioxidants such as vitamins appear to be of great importance for the control of the effects of reactive oxygen species. In our study, the levels of all four antioxidant vitamins were significantly lower in MS patients compared to controls. One possible explanation for the low levels of vitamins is that MS may have caused decreased dietary intake vitamins as was partly in the study of Warren (1982). This seems unlikely, as none of the patients had any evidence of nutritional deficiency. A more likely explanation would be that these vitamins are consumed at an increased rate as defense mechanisms of the organism against the ongoing oxidative burden. This explanation may well be partly satisfactory as correlation was observed between the serum levels of vitamins and of TBARS. However the results of this study do not elucidate a definitive cause–effect relationship between the antioxidant vitamins and oxidative stress in MS. Alpha tocopherol is the major lipid-soluble antioxidant which is capable of breaking the lipid peroxidation chain reaction in cell membranes, thereby preventing the formation of

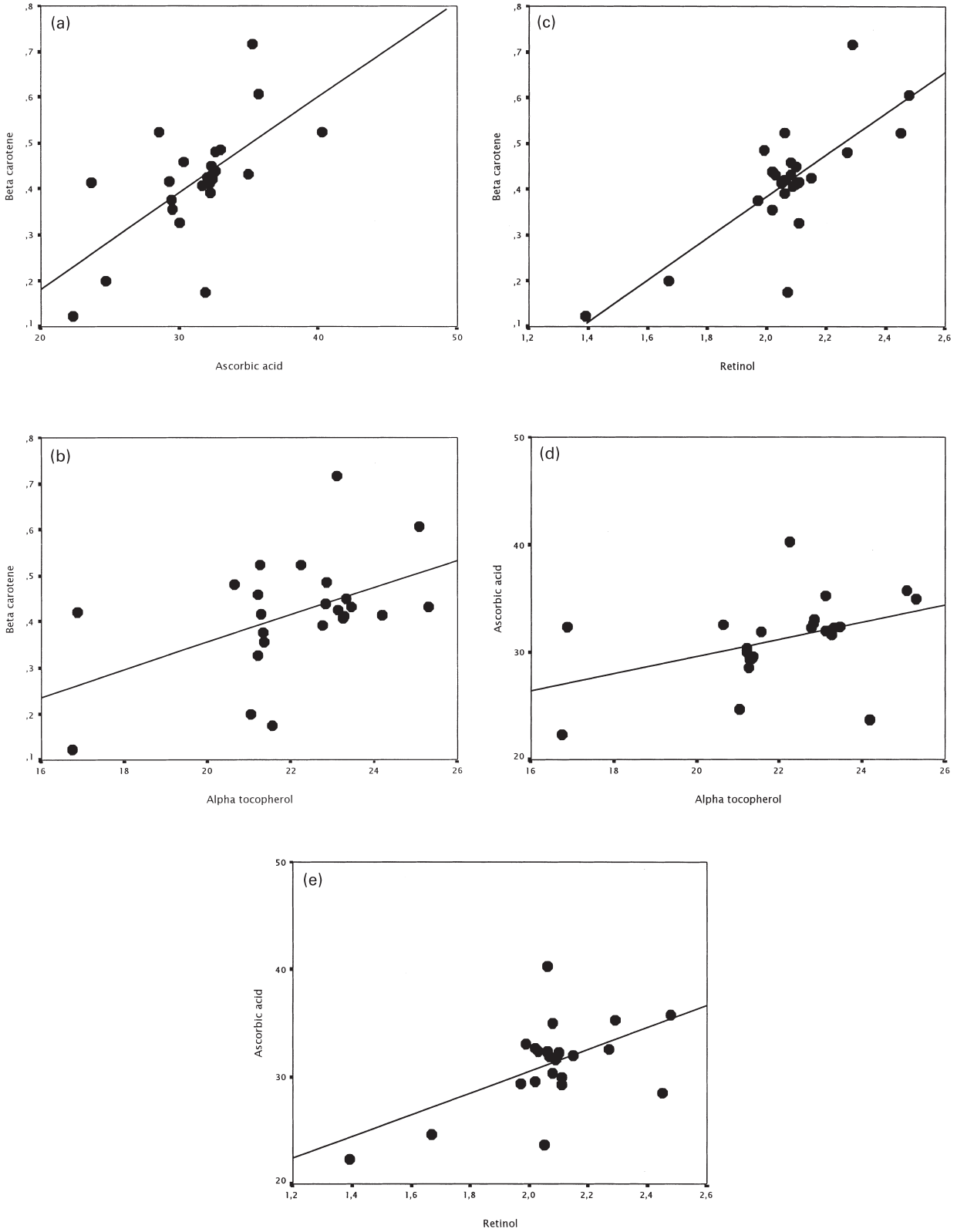

FIGURE 1 Correlations among the antioxidant vitamins in MS patients. (a) Correlation between serum beta-carotene and ascorbic acid ($r^2 = 0.727$, $p < 0.05$). (b) Correlation between serum beta-carotene and alpha tocopherol ($r^2 = 0.689$, $p < 0.05$). (c) Correlation between serum beta-carotene and retinol ($r^2 = 0.774$, $p < 0.05$). (d) Correlation between serum ascorbic acid and alpha tocopherol ($r^2 = 0.714$, $p < 0.05$). (e) Correlation between serum ascorbic acid and retinol ($r^2 = 0.779$, $p < 0.05$).

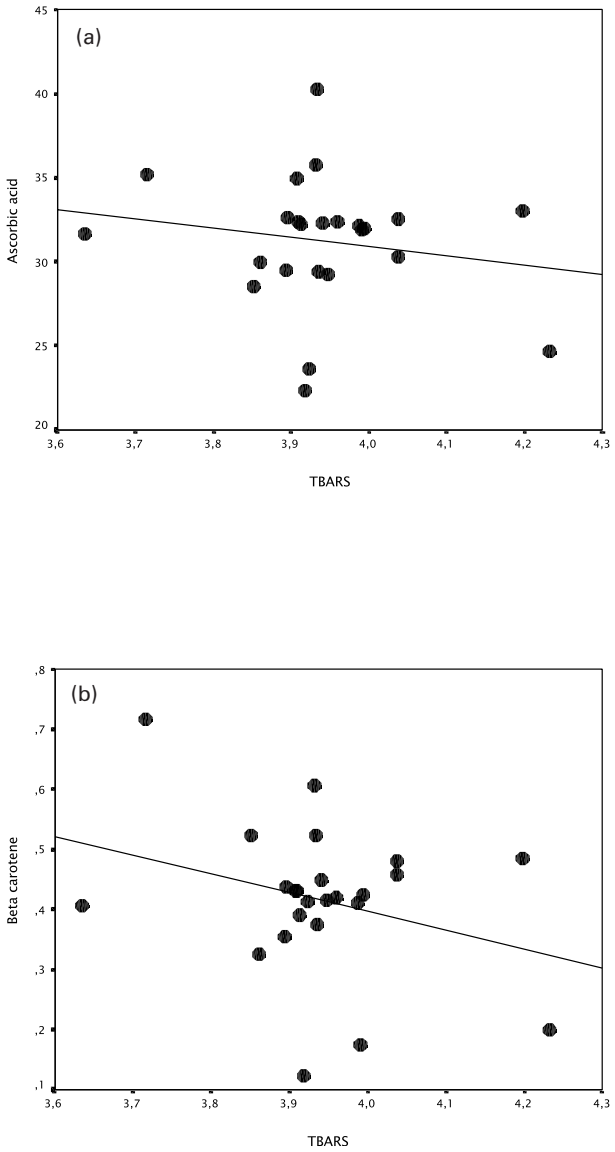

FIGURE 2 Correlation between the serum levels of ascorbic acid or beta-carotene and lipid peroxidation products (estimated as TBARS). (a) Correlation between the serum levels of ascorbic acid and TBARS ($r^2 = 0.689$, $p < 0.05$). (b) Correlation between the serum levels of beta-carotene and TBARS ($r^2 = 0.692$, $p < 0.05$).

lipid hydroperoxides derived from polyunsaturated fatty acids. Beta-carotene is an avid quencher of singlet oxygen. Retinol is less important as an antioxidant vitamin. The mechanism of its antioxidant action presumably is similar to that of beta-carotene. Ascorbic acid is the first and major line of antioxidative defense against radicals in the aqueous phase and prevents any lipid peroxidation in blood as long as it is present (Gey, 1998). Further, ascorbic acid is equally crucial for the transfer of radical equivalents from lipid phases into aqueous compartments, i.e. also for the recycling of vitamin E (Zhang and Omaye, 2001). In short-term experiments with intact ascorbic acid deficient animals, the increased susceptibility towards lipid peroxidation can be controlled not only by ascorbic acid rectification but also pretreatment with either alpha tocopherol or beta-carotene which suggest synergism in the antioxidant defense system (Gey, 1998).

A number of studies support the role for oxidative stress in MS. These include increased serum peroxide levels in MS relative to control. Patients with MS in acute exacerbation exhibit significantly higher levels of pentane and hexane (products of lipid peroxidation) in expired breath compared to either MS patients in remission or control subjects (Toshniwal and Zarling, 1992). Moreover, recent clinical and animal studies suggest that NO and its reactive derivative peroxynitrite are implicated in the pathogenesis of MS (Bö *et al.*, 1994). In accord with our study, de Bustos *et al.* (2000a) showed that MS patients had not significantly lower ascorbic acid, alpha tocopherol, and beta-carotene and retinol levels than control. Karg *et al.* (1999) reported that the plasma lipid peroxides expressed in terms of TBARS levels were increased as well as decreased in the level of tocopherol, but not of retinol. It was also reported that in the demyelinating plaques, there were decreased glutathione and alpha tocopherol concentrations, normal ascorbic acid and increased uric acid levels (Jimenez-Jimenez *et al.*, 1998). It has been reported that cerebrospinal fluid alpha tocopherol levels and serum coenzyme Q_{10} levels were normal (Wong *et al.*, 1993; de Bustos *et al.*, 2000b). In addition, glutathione-peroxidase activity was decreased and glutathione-reductase activity was increased in the cerebrospinal fluid. The findings of those studies as well as the present study may support the idea that there is in fact a strong relationship between antioxidant vitamins and oxidative stress although there are some other factors involved in the pathogenesis of MS.

The observation of very strong correlations among three antioxidant vitamins (beta-carotene, alpha tocopherol and ascorbic acid) in our study strongly suggest that their involvement is most likely mediated via the same mechanisms. Our study raises the interesting question of whether or not vitamin supplementation could be of any value in prevention or treatment of MS.

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