RESEARCH ARTICLES

LIPID PEROXIDATION AND TOCOPHEROL IN BLOOD OF PATIENT WITH PARKINSON'S DISEASE

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ABSTRACT

Free radicals and oxidative stress have been suggested in the pathogenesis of Parkinson's disease (PD). Free radicals exert their cytotoxic effect by peroxidation of lipid membrane resulting in the formation of malondialdehyde (MDA). This condition has been found to occur in the substantia nigra (SN). The present study was designed to investigate whether oxidative stress extended beyond the CNS. Therefore, the levels of lipid peroxidation and tocopherol in plasma and erythrocytes of the patients were measured. Twelve idiopathic PD patients with early or non-motor fluctuation (NF-PD), ten idiopathic patients with severe motor fluctuations (MF-PD), and seventeen age-matched healthy subjects (NM), were included in this study. The lipid peroxidation was determined by thiobarbituric acid test and reported as thiobarbituric acid reactive substance (TBARS), and the level of tocopherol was determined by HPLC. The results showed that the plasma TBARS was significantly increased in MF-PD patients compared to control group (0.721 ± 0.124 and 0.538 ± 0.124 nmol/ml respectively, $p < 0.05$) but not NF-PD group (0.662 ± 0.156 nmol/ml). There was no significant difference between NF-PD patients and control group. The erythrocyte susceptibility to hydrogen peroxide induced-autoxidation, as well as the level of tocopherol in plasma and erythrocytes were not significantly different among the three groups. The correlation between ages of the patients, the duration of disease and those markers were not evident in this study, but there was positive relationship between severity of the disease manifested as motor fluctuation and plasma TBARS. Our finding clearly indicated that oxidative stress was present in systematic circulation of PD patients with severe motor fluctuations and it was tissue specific.

Keyword : Parkinson's disease, oxidative stress, lipid peroxidation , tocopherol,

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ปฏิกิริยาออกซิเดชันของไขมันและ tocopherol ในเลือดของผู้ป่วยปาร์กินสัน

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บทคัดย่อ

อนุมูลอิสระและภาวะเครียดออกซิเจนเป็นปัจจัยหนึ่งที่นำไปสู่การเกิดพยาธิสภาพของโรคปาร์กินสัน ในสมองส่วน substantia nigra อนุมูลอิสระนำไปสู่การเกิดปฏิกิริยาออกซิเดชันของไขมันที่เยื่อหุ้มเซลล์ทำให้เกิดการสร้างสาร malondialdehyde การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาถึงภาวะเครียดออกซิเจนในระบบประสาทส่วนกลาง โดยติดตามระดับของการเกิดออกซิเดชันของไขมันและ tocopherol ในน้ำเลือดและเม็ดเลือดแดงของผู้ป่วยปาร์กินสัน อาสาสมัครที่เข้าร่วมในการศึกษาประกอบด้วย ผู้ป่วยปาร์กินสันที่ไม่ทราบสาเหตุมีอาการไม่รุนแรง ยังไม่ได้รับยาลีโวโดปาหรือได้รับยาลีโวโดปาและดอบสนองต่อยาดี (NF-PD) จำนวน 12 คน ผู้ป่วยที่มีอาการรุนแรงได้รับยาลีโวโดปาและดอบสนองต่อยาลดลงหรือมีอาการเคลื่อนไหวที่ผิดปกติอันเนื่องมาจากการใช้ยาลีโวโดปา (MF-PD) จำนวน 10 คน และอาสาสมัครที่มีสุขภาพดีอายุใกล้เคียงกับผู้ป่วย (NM) จำนวน 17 คน ติดตามการเกิดออกซิเดชันของไขมันโดยวัดระดับสารที่ทำปฏิกิริยากับกรดไทโอบาบิทูริค (TBARS) และวัดระดับสาร tocopherol โดยใช้ HPLC จากผลการทดลองพบว่า ในผู้ป่วยกลุ่ม MF-PD มีค่าของระดับ TBARS สูงขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม (NM) แต่ไม่พบความแตกต่างของค่าดังกล่าวระหว่างกลุ่มของ MF-PD และ NF-PD รวมทั้งระหว่างกลุ่ม NF-PD และกลุ่ม NM ระดับของ tocopherol ในน้ำเลือดและเม็ดเลือดแดงและโอกาสที่เม็ดเลือดแดงจะถูกออกซิเดชันโดยไฮโดรเจนเพอร์ออกไซด์ ไม่มีความแตกต่างกันทั้ง 3 กลุ่ม มีความสัมพันธ์ระหว่างความรุนแรงของโรคซึ่งบ่งชี้โดยความผิดปกติของการเคลื่อนไหวกับระดับ TBARS ในน้ำเลือด แต่ไม่พบความสัมพันธ์ใด ๆระหว่างอายุของผู้ป่วย, ระยะเวลาของการเกิดโรค กับตัวบ่งชี้ทุกค่า การศึกษาครั้งนี้ชี้ให้เห็นว่ามีภาวะเครียดออกซิเจนในระบบไหลเวียนของผู้ป่วยปาร์กินสันระยะรุนแรงที่มีความผิดปกติของการเคลื่อนไหวมาก โดยภาวะนี้พบจำเพาะเจาะจงในเนื้อเยื่อบางชนิด

คำสำคัญ: ปาร์กินสัน, ภาวะเครียดออกซิเจน, ปฏิกิริยาออกซิเดชันของไขมัน, tocopherol

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized mainly by a loss of nigrostriatal dopamine neuron[1]. The causative factor responsible for the neuronal loss remains unknown. However, results from recent studies have demonstrated that oxidative stress could be a major cause of nigral cell death in PD[2-4]. It is assumed that the loss of nigral cells is a direct consequence of excessive oxidative stress due to an excess of generation or a defective removal of free radicals and reactive oxygen species (ROS). These ROS are normally generated in dopamine metabolism[5,6] and able to react with polyunsaturated fatty acids of cell membrane, which ultimately destroy the cell membrane and neuron. Several experiments have demonstrated excessive concentrations of the lipid peroxidation products including malondialdehyde (MDA)[7] lipid hydroperoxide[8], and 4-hydroxynonenal [9] in the postmortem substantia nigra (SN) of PD patients. Within the last few years, many lines of evidence demonstrated that oxidative stress also occurred in systemic circulation [10-14]. Most studies have reported an increase in the concentration of MDA in serum[15] , plasma and erythrocytes[11] of patients with PD, while, others reported dissimilar result[16]. The exact significance of these finding is unclear and still controversial. However, these have led some investigators to recommend treating patients with antioxidant tocopherol.

Alpa-tocopherol is the principal lipid soluble, chain breaking antioxidant and radical scavenger in human tissue[17]. It has been postulated that antioxidants may have a role in the prevention and/or management of PD[18]. However, alteration of tocopherol concentrations in the SN and serum of PD patients has not been reported[19,20]. The Deprenyl and Tocopherol Antioxidant Therapy of Parkinson (DATATOP) trial demonstrated that tocopherol could not delay the disability associated with the need for levodopa therapy and could not lessened motor impairment in patients with early, otherwise untreated PD[21].

Therefore, the purpose of this study is to investigate whether there is any difference in the degree of oxidative stress reflected as changes in the level of lipid peroxidation and tocopherol concentration occur in blood of patients with different severity stages of the Parkinson's disease.

MATERIALS AND METHODS

Subjects

The experiments were performed on three groups of subjects, the idiopathic PD patients with non-motor fluctuation (NF), those with motor fluctuation (MF) and healthy subjects as control (NM). The diagnosis of parkinsonism was based on the presence of at lease two or three cardinal features of PD, i.e. resting tremor, bradykinesia, and rigidity. The patients were not included in this study if the causes of the disease were identified. Twelve patients were in the early stage. They did not have clinically appreciable fluctuations in motor performance while receiving levodopa-carbidopa nor never been treated with levodopa or other dopaminergic agonists, and they were grouped as non-motor fluctuated Parkinson's disease (NF-PD). Ten patients manifested abnormal movements. These mobility complications occurred as predictable "wearing-off" fluctuations, unpredictable "on-off " fluctuations or dyskinesia, and they were grouped as motor fluctuated Parkinson's disease (MF-PD). Seventeen healthy and age matched normal volunteers were included as control group in the study. The protocol was reviewed and approved by The Committee in Human Rights Related to Human Experimentation of Mahidol University. All participants both healthy volunteers and PD patients understood the scope and objective of this study before giving the informed consents. They were interviewed comprehensively about their medical history and drug history and were asked not to take any medication or vitamin supplement, except their daily antiparkinsonian drug, at least 1 month before blood drawing. All patients and control subjects underwent a complete blood count (CBC) and serum chemistry profile study to confirm the absence of any clinically significant medical complication. PD patients were received a complete physical examination, evaluated by using the Unified Parkinson's Disease Rating Scale (UPDRS) and determined the stage by using the Hoehn and Yahr scale[22]. The demographics of all subjects were shown in Table 1.

To obtain clinical CBC and blood chemistry profile data, fasting blood was drawn in the morning; and glucose, triglyceride, chloresterol, liver function and renal function were routinely analyzed by Department of Pathology, Ramathibodi Hospital. The blood

Table 1 Characteristics of subjects

Group	Male : Female	Age (years)	Age at onset (years)	Stage [a]	Dose of levodopa (mg/day)	Duration of (years)		
						PD	Tx	MF
NM (N=17)	7:10	56 ± 6 (42-60)	-	-	-	-	-	-
NF-PD (N=12)	9:3	55 ± 7 (41-66)	52±8 (40-65)	1.33 (1-2)	220 ± 130 (100 – 450)	4 ± 3 (1-11)	2.91± 3.23 (0.17-11)	-
MF-PD (N=10)	8:2	60 ± 10 (48-75)	48 ± 9 (37-61)	3 (2-5)	628.75 ± 249 (400 – 1200)	13 ± 4 (8 - 20)	11.4± 4.14 (7-20)	4.63±3.42 (0.33-10)

Values are expressed as mean ± SD. The numbers in parentheses indicate the range of values.
a = stage determined by Hoehn and Yahr scale [22]; NM = normal volunteer; NF = Parkinson's disease with non-motor fluctuation; MF = Parkinson's disease with motor fluctuation; PD = Parkinson's disease; Tx = treatment; MF = motor fluctuation

sample was centrifuged at 2500 x g for 15 minutes at 4° C. Plasma was removed and determined for the level of thiobarbituric acid reactive substance (TBARS) and tocopherol. Erythrocytes were prepared and determined for the level of tocopherol and their susceptibility to hydrogen peroxide induced-autoxidation.

Measurement of lipid peroxidation in plasma

The lipid peroxidation product in plasma was determined by thiobarbituric acid test (TBA test) and expressed as TBARS[23]. One milliliter of sample or standard solution was mixed with 50 µl of 100 µM butyl hydroxy toluene (BHT), 1.0 ml of 10 % trichloroacetic acid, 0.5 ml of 5 mM EDTA, 0.5 ml of 8.1% sodium dodecyl sulfate and 1.5 ml of 0.6 % thiobarbituric acid. Control experiments were performed by using the same amount of normal saline solution instead of sample or standard solution. The reaction mixtures were well mixed and incubated in water bath at 90°C for 1 hour. After cooling, they were centrifuged at 2500 x g for 10 minutes. Supernatant was collected and transferred to a cuvette. The TBARS complex had characteristic fluorescence excitation and emission maxima at 515 nm and 553 nm, respectively. The fluorescence intensity of the product was determined at 553 nm by a spectrofluorometer. 1,1,3,3 Tetra-ethoxypropane was used to serve as a standard.

Determination of tocopherol

Level of tocopherol in plasma was determined by a reverse phase high performance liquid chromatography (HPLC)[24]. A NovaPak C18 (4 mm; 3.9 x 150 mm) column was used for the separation of tocopherol. The HPLC grade (100%) methanol was used as mobile phase. Flow rate was set at 1 ml/min and the pumping pressure was 1,000 psi. This condition was performed at ambient temperature. The fluorescence detector was operated at the wavelengths of excitation (295 nm) and emission (370 nm). A 500 µl of plasma was extracted with 1.0 ml of sodium dodecyl sulfate (0.1M), 2.0 ml of ethanol and 2.0 ml of hexane. The supernatant was collected and evaporated to dryness by nitrogen gas and redissolved in 500 µl of ethanol. Five µl aliquot of samples or standards was injected into the column.

Susceptibility to autoxidation of erythrocytes

Susceptibility to autoxidation of erythrocytes was determined by the procedure of Stocks et al.[25]. Freshly withdrawn

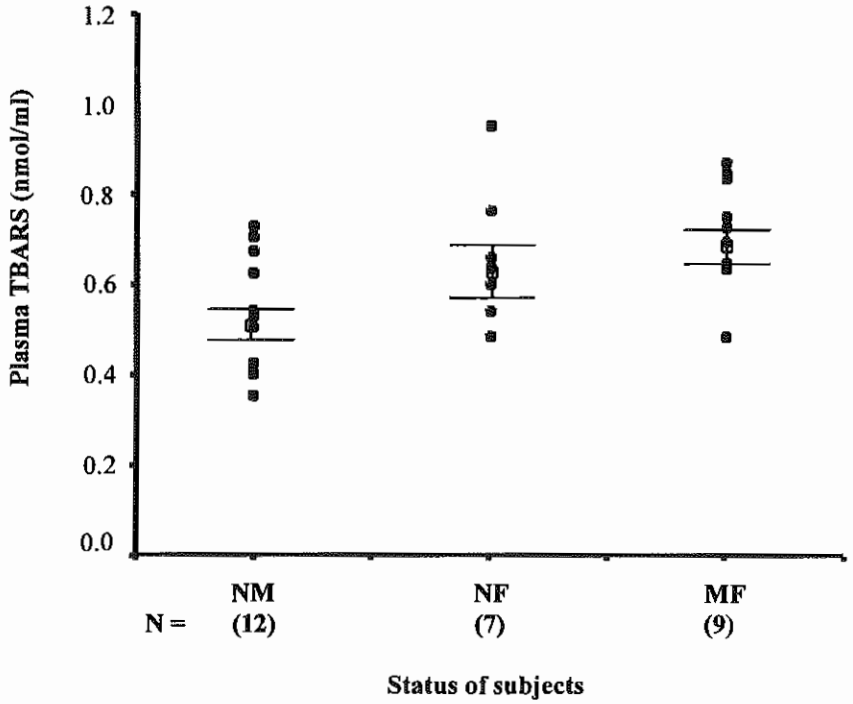

Figure 1 Plasma TBARS level in normal volunteers (NM) and Parkinson's disease patient with non motor fluctuation (NF) and with motor fluctuation (MF). Dots represent values of enzyme activity in individual subjects, bars indicate standard error of the means and squares are mean values. The difference between NM and MF-PD patients was significant ($P < 0.05$). Values in NF-PD patients were not significantly different from NM subjects.

heparinized blood was spun. The plasma was aspirated and replaced by an approximately equal volume of azide buffer. The cells were resuspended. Two ml cell suspension was diluted with 8 ml azide buffer and after mixing the cell suspension was spun. The supernatant and the remainder of the buffy coat were removed. Five ml of azide buffer was added to the packed cell and the cell suspension was mixed. The number of red blood cell in the suspension and hemoglobin was determined by H_3-Technicon cell analyzer. Three ml of this suspension was transferred to a glass boiling tube and equilibrated in a 37°C shaking water bath for 10 minutes. Three ml of 10 mM hydrogen peroxide solution was added by allowing the solution to run down the side of the tube (zero time). The mixture was then incubated at 37°C for 2 hr. 1,1,3,3, Tetraethoxypropane was used to serve as a standard.

MDA estimation and calculation

Three ml of the cell suspension was added to 2 ml TCA-arsenite solution. The mixture was spun for 10 minutes at 2500 x g and 3 ml of the supernatant was transferred to a centrifuge tube and 1 ml of TBA solution was added. The mixture was incubated for exactly 15 minutes in a boiling water bath in a closed system. The tube was cooled. The absorption of the mixture at 532 and 600 nm was measured by a recording spectrophotometer.

Statistical analysis

The data were analyzed by SPSS version 7.52 and expressed as mean ± SD. Mann-Whitney U test was used to compare between normal and each group of PD patients. The association between two parameters was computed by Spearman's rank correlation test. P value ≤ 0.05 was considered as significant difference.

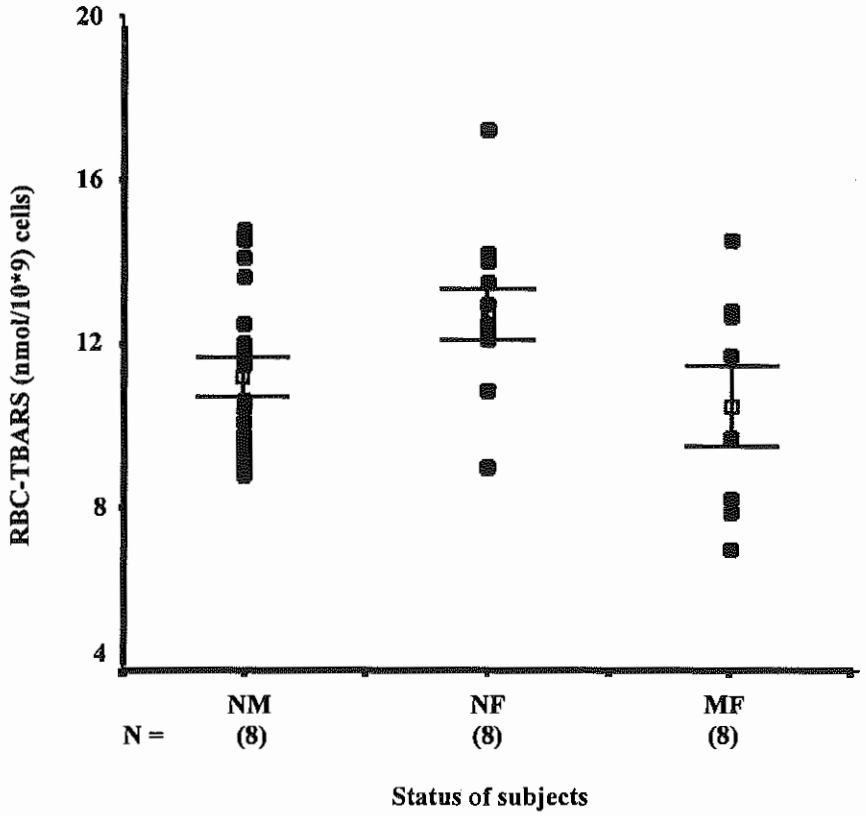

Figure 2 The levels of erythrocyte TBARS in normal volunteers (NM), Parkinson's disease patients with non-motor fluctuation (NF) and Parkinson's disease with motor fluctuation (MF). Dots represent values of erythrocytes TBARS levels in individual subjects, bars indicate standard error of the means, and squares are mean values. Values in both groups of patients were not significantly different from normal volunteers.

RESULT

Lipid peroxidation

The mean plasma level of TBARS was significantly higher in MF-PD group (0.72 ± 0.12 nmol/ml) compared to healthy subjects (0.54 ± 0.12 nmol/ml, $p < 0.05$, figure 1) but it was not significantly different form NF-PD group (0.66 ± 0.16 nmol/ml). There were no correlation between plasma TBARS and age of the subject (MF: $r = 0.117$, $p = 0.764$; NF: $r = 0$, $p = 1$; NM: $r = -0.035$, $p = 0.913$) or duration of disease in both groups of PD patients (MF: $r = 0.126$, $p = 0.747$; NF: $r = -0.400$, $p = 0.60$).

Erythrocyte susceptibility to autoxidation

There were no significant difference in erythrocyte susceptibility to autoxidation among three groups of subjects (NM = 11.262 ± 2.039, NF = 12.828 ± 2.068, MF-PD = 10.566 ± 2.764 nmol/10*9 cells figure 2). No correlation was observed between the level of MDA and age of the subject (MF: $r = -0.548$, $p = 0.160$; NF: $r = 0.359$, $p = 0.382$; NM: $r = -0.129$, $p = 0.622$) or duration of the disease from all tested groups (MF: $r = -0.0132$, $p = 0.977$; NF: $r = 0.482$, $p = 0.227$).

The level of vitamin E in plasma and erythrocytes

There were no significant differences in the level of vitamin E in plasma among the three groups of subjects. (NM = 13.530 ± 2.658, NF = 15.274 ± 7.933, MF = 14.01 ± 7.389 μg/ml, figure 3) and the level of vitamin E in erythrocyte (NM = 2.15 ± 0.471, NF = 2.334 ± 1.779, MF = 3.889 ± 2.067 μg/g Hb, figure 4). There were no correlation between these parameters and age of subjects (plasma vitamin E –MF : $r = -0.084$, $p = 0.831$; NF : $r =$

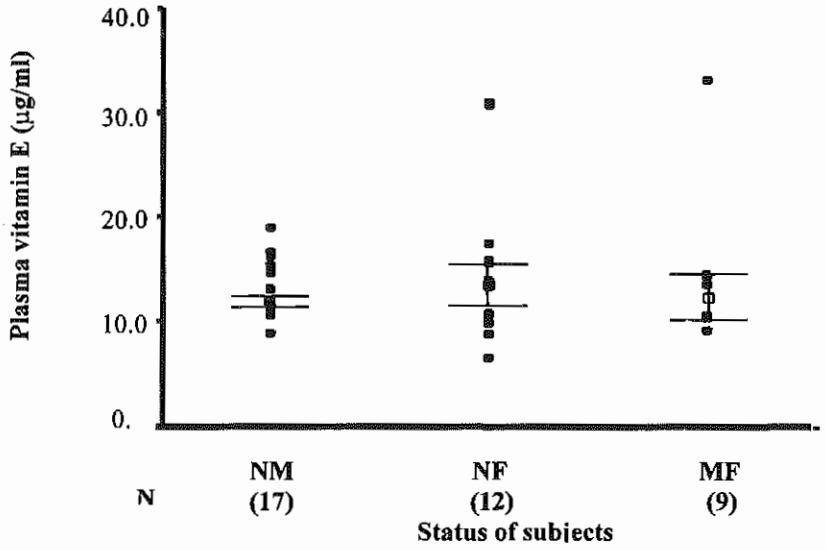

Figure 3 Plasma vitamin E levels in normal volunteers (NM), Parkinson's disease patients with non-motor fluctuation (NF) and Parkinson's disease with motor fluctuation (MF). Dots represent values of vitamin E levels in individual subjects, bars indicate standard error of the means and squares are mean values. Values in both groups of patients were not significantly different from normal volunteers.

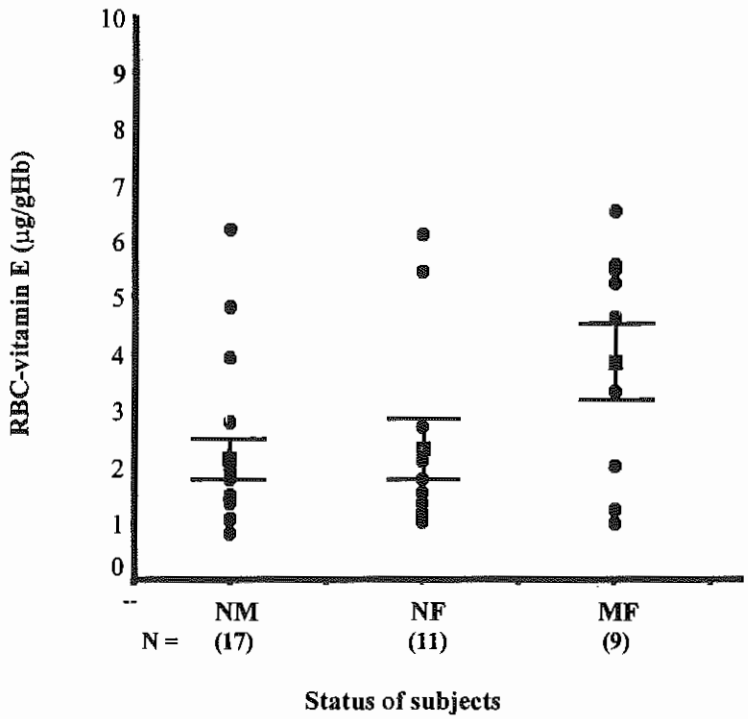

Figure 4 The levels of erythrocyte vitamin E in normal volunteers (NM), Parkinson's disease patients with non-motor fluctuation (NF) and Parkinson's disease with motor fluctuation (MF). Dots represent values of vitamin E levels in individual subjects, bars indicate standard error of the means, and squares are mean values. Values in both groups of patients were not significantly different from normal volunteers.

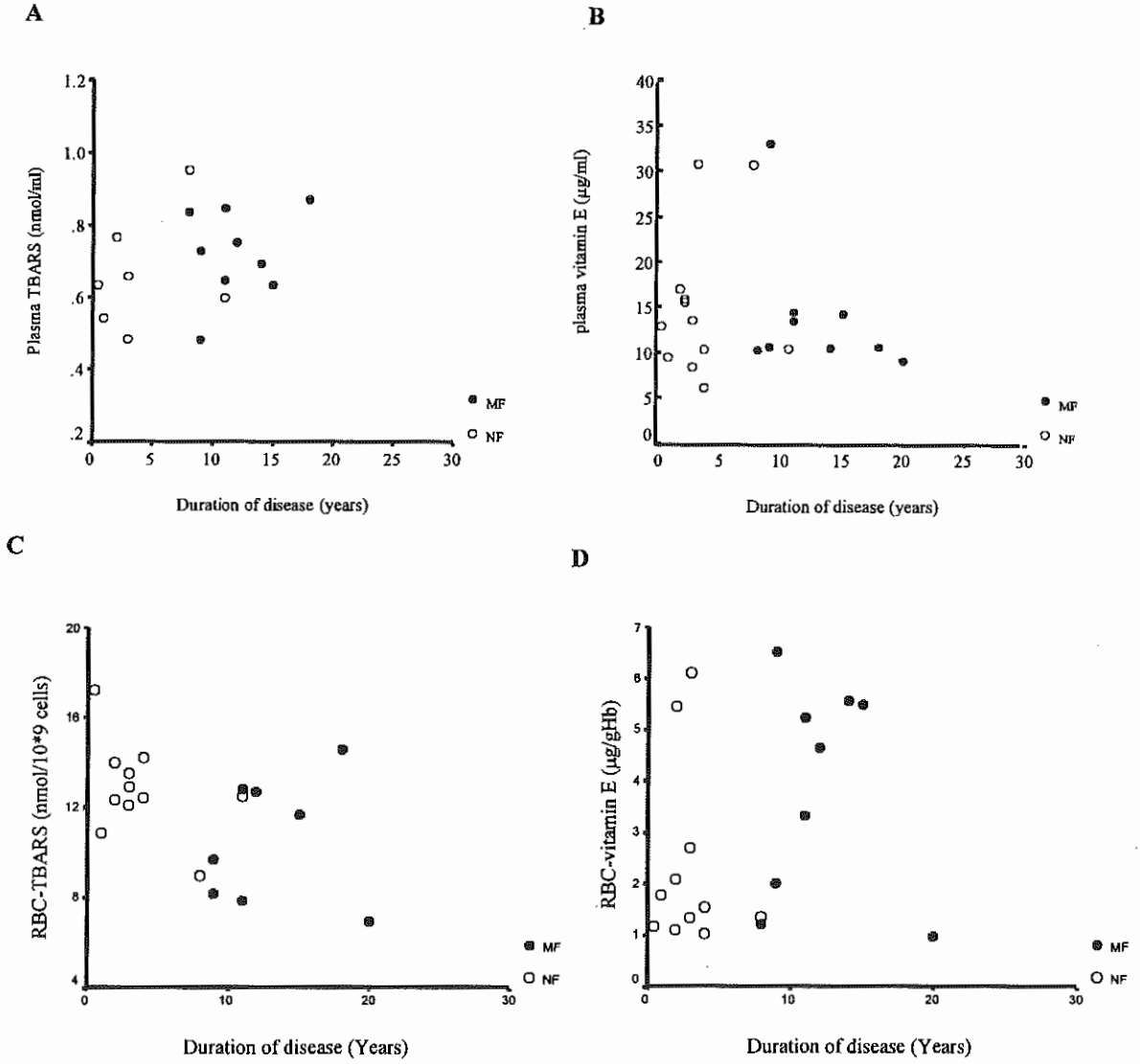

Figure 5 Relationship between oxidative markers and duration of disease in Parkinson's disease patients. The lack of correlation was observed in (A) plasma TBARS, (B) plasma vitamin E, (C) erythrocyte susceptibility to hydrogen peroxide, and (D) erythrocytes vitamin E. Closed circles represent values of enzyme activity in individual MF-PD patients and open circles represent values of enzyme activity in individual NF-PD patients.

-0.286, p = 0.535; NM: r = 0.138, p = 0.596; RBC-vitamin E – MF: r = -0.008, p = 0.983; NF: r = 0.338, p = 0.309) or duration of disease in both groups of PD patients (plasma vitamin E - MF : r = 193, p = 0.618; NF : r = 0.236, p = 0.610, RBC- vitamin E MF: r = 0.050, p = 0.897; NF: r = -0.088, p = 0.797).

DISCUSSION

Our data demonstrated that the plasma TBARS was significantly increased in MF-PD patients but not in NF-PD patients compared to NM group. Oxidative stress has long been known to cause lipid peroxidation

which is associated with pathophysiological events in a variety of diseases. It has been postulated that free radicals and aldehydes generated during the process may be responsible for these effects because of their ability to damage cellular membrane protein and DNA[26]. TBARS is commonly recognized as a marker of lipid peroxidation[27]. The significant increase in plasma TBARS in MF-PD patients suggest a marked increase in oxidative stress in late complicati of the disease.The motor abnormalities represent the decreased striatal storage of dopamine due to drastic loss of dopaminergic nerve terminals[28]. Parkinsonism begins to appear when striatal dopamine concentrations are reduced by 80%[29], and a more severe state of disease is associated with a 90 to 98 % reduction in striatal dopamine [29,30].

The result of this study not only support the association of oxidative stress in pathogenesis of this disease but also suggest that in the late stage the oxidative marker can be detected in peripheral tissues, since significant increase in the level of TBARS is found in blood of PD patients with motor fluctuation. These can be implied that lipid peroxidation product occurring during PD period accumulated in plasma. The study in cell culture demonstrated that MDA was extensively bound to serum albumin presented in the media[31]. Thus, it seems to be possible that this event may also occur in plasma.

It has been demonstrated that the levels of blood lipid peroxide in the elderly were significantly higher than in the young group[32]. However, the data of our study showed no correlation between the levels of TBARS and ages of the patients or duration of the disease. Since the ages of the patients enrolled in this study are between 41-66 and 48-75 years old in NF-PD and MF-PD, respectively, it may not be possible to reveal any significant difference in their physiological functions in these age ranges unless greater number of patients are enrolled.

The present data are in substantial agreement with many other studies [7,8,15,16]. However, Ahlskog et al[16] reported no significant difference in mean serum TBARS in levodopa treated and untreated patients with PD, compared to normal control. The discrepancies may be due to the difference in patient selection. In our experiment, we classified PD patients as NF-PD and MF-PD, according to the degree of disease severity.

Since aldehyde which occurrs as a product of oxidative damage is a causative agent in certain pathological conditions[27], therefore, the importance of antioxidant defense system in protecting biological tissue against ROS mediated peroxidation has promoted investigation into the prophylactic use of high dose antioxidant supplement [33].

Vitamin E plays an important role as an antioxidant for unsaturated lipids and in maintaining the integrity and stability of biologic membrane[34,35]. Our result showed that the levels of vitamin E in plasma and erythrocyte were not significantly different in both groups of PD patients when compared to controls. These findings are consistent to previous report[36-38]. The relationship between PD and plasma vitamin E concentration remains unclear. Some authors, based on a retrospective interview study, have suggested that PD patients in early life might have taken food with lower vitamin E content than did controls[39]. These studies migth be unreliable because the interviews were retrospective, covering a long period of time and only a few of the investigated foodstuffs differed significantly between PD and control groups. However, the study of the levels of vitamin E in brains of PD showed that they were not altered from normal level [19].

The results from multicenter controlled clinical trial involving 800 patients receiving vitamin E either with placebo or with selegiline (a monoamine oxidase type B inhibitor) have been reported [21]. No beneficial effects of vitamin E were found and there appeared to be no interaction between vitamin E and selegiline. The failure of vitamin E to influence the progression of PD in this study does not necessarily mean that antioxidant is not effective. Vitamin E is a secondary antioxidant that halts the chain reaction of lipid peroxidation and may be less effective than primary antioxidant that prevent the formation of ROS and the initiation of lipid peroxidation.

Fahn[40] reported preliminary data that the use of high doses of vitamin E (3,200 IU/d) together with vitamin C (3,000 mg/d) may delay the need for levodopa by 2 to 6 years. This may not be attributable to vitamin E alone, since vitamin C promotes the reformation of vitamin E from vitamin E radical and it also possesses antioxidant activity[41]. In addition, glutathione can regenerate vitamin C[42,43]. Therefore, although vitamin E may scavenge free radicals, its level is preserved at the expense of other

plasma TBARS as systemic manifestation of oxidative marker for the disease. The severity of the disease and tissue specificity also has some impacts on this marker.

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