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Increased oxidative stress in patients with essential thrombocythemia

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Abstract. – BACKGROUND: Essential thrombocythemia (ET) is a clonal disease in which thrombotic and hemorrhagic complications are common. Our aim in this study was to investigate whether oxidative stress in ET patients increased compared to healthy volunteers and to investigate whether there is a relationship between vascular events and oxidative status parameters in ET patients.

PATIENTS AND METHODS: We determined the serum levels of oxidative status parameters, such as total oxidative status (TOS), total antioxidant status (TAS), oxidative stress index (OSI) and malondialdehyde (MDA) in ET patients. Forty-three ET patients (20 males, 23 females) and 20 healthy volunteers were enrolled. Oxidative status parameters of the patients were compared with those of the controls at time of diagnosis and at 6th-month follow-up. Additionally, oxidative status parameters of patients with ET with a history of vascular event were compared with patients without a vascular event history during diagnosis.

RESULTS: Rises in TOS, OSI, and MDA were statistically significant in the patients group; however, the TAS value was significantly lower compared to the control group. Furthermore, TOS was significantly higher in patients with history of vascular event compared to the patients without such a history. Following therapy, OSI and MDA values were significantly reduced in the patient group compared to the pre-treatment values.

CONCLUSIONS: Our findings reveal that although oxidative stress parameters were increased, compensative total antioxidant status was significantly reduced in ET patients. Furthermore, TOS values were significantly high in patients with a history of vascular event.

Key Words:
Essential thrombocythemia, Vascular event, Oxidative stress, Antioxidant status.

Introduction

Essential thrombocythemia (ET), a chronic myeloproliferative disease, is a clonal disorder of the hematopoietic stem cells that may be associated with thrombotic and hemorrhagic complications. The annual incidence is 1-2.5/100,000 and the disease is common between the ages of 50-70[1]. Cytoreductive therapy is generally administered to high-risk ET patients. Thrombotic complications, the major cause of mortality and morbidity in ET patients within 27 months of diagnosis, is 24% in untreated patients[2]. Vascular events and ischemia may be identified in some of these patients at the time of diagnosis, although they may also develop during treatment or in the follow-up period without treatment. However, there are still no definitive standard criteria regarding which characteristics lead to these vascular events and when they develop.

Oxidative stress is associated with changes in the pro-oxidant and antioxidant balance in favor of pro-oxidants[3]. Viability and growth of cells in an environment containing oxygen are not possible without defense mechanisms consisting of enzymatic and non-enzymatic antioxidant components (the antioxidant system). Studies have demonstrated increased oxidative status in conditions including diabetes mellitus (DM)[4], chronic renal failure[5] and iron deficiency anemia[6]. However, living organisms develop antioxidant defense in order to avoid the harmful effects of increased free radicals as a response to the oxidative stress.

Measuring each antioxidant separately is difficult due to time loss, laboratory burden, high cost, the need for complex techniques and the interaction between different antioxidants in the

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serum[7,8]. Using newly developed methods, all of these antioxidants can be measured in serum more easily, with lower costs in a very short period of time as a single value known as total antioxidant status (TAS). In uremic patients, oxidative stress has been shown to be an important cofactor contributing to endothelial dysfunction, atherosclerosis and uremic hypertension[9,10].

The pathological role of oxidative stress in vascular diseases has been well described[11]. Since vascular events are increased in ET patients, we aimed to assess whether oxidative stress was also increased in these patients.

Patients and Methods

Forty-three patients admitted to the Hematology Clinic of the Trabzon Kanuni Teaching and Research Hospital, Turkey, and diagnosed with ET according to the diagnostic criteria of the World Health Organization (WHO-2008) and 20 healthy volunteers with similar demographic characteristics were included. The patients signed consent forms, and approval was granted by the local Ethics Committee (approval number: 29-2011). The patient group consisted of 20 males and 23 females, with a mean age of 63.3±1.9 years (range: 28-98). Patients with ET were classified into risk groups based on age, thrombosis history and thrombocyte counts; high risk (age >60 years, prior thrombosis, platelets >1500x10^3/μl), intermediate risk (age 40-60 years), and low risk (age <40 years)[12]. Since the treatment regimen varied according to risk groups, treatment protocols are set out below.

Overall, 29 patients (67.4%) were in the high-risk and 14 in the moderate/low-risk categories. High-risk patients were given hydroxyurea or anagrelide and low-dose aspirin (100 mg/day), while moderate/low-risk patients received only low-dose aspirin.

Exclusion Criteria

Patients meeting criteria which might lead to oxidative stress, such as alcohol use, smoking, intravenous drug use, pregnancy, antioxidant use (Vitamin E, β-carotene, ascorbic acid, glutathione, probucol), fish oil or iron supplementation, and patients with human immunodeficiency virus infection, rheumatoid arthritis, cirrhosis, active infection or malignancy were excluded.

Blood Collection

Peripheral venous blood samples were drawn from the healthy volunteers and the patients at time of diagnosis and at the end of 6 months of treatment. Tubes containing gel were used in order to separate serum for assessing oxidative status parameters. Blood samples were centrifuged at 3000 rpm for 10 min, and serum samples were stored in a deep-freezer (–30 °C) until analysis for serum oxidative status markers. The serum samples were removed from the freezer and thawed before measurements. They were then analyzed for TAS, TOS and MDA, and the OSI value was calculated. Tubes containing EDTA were used in the evaluation of hematological parameters and JAK2V617F mutation.

Serum Malondialdehyde Activity Assay

Lipid peroxidation in human serum samples was determined as MDA concentration using the method described by Yagi[13]. Briefly, 0.3 mL of serum was mixed with 2.4 mL of N/12 H_2SO_4 and 0.3 mL of 10% phosphotungstic acid. After being allowed to stand at room temperature for 5 min, the mixture was centrifuged at 1600 g for 10 min. Supernatant was discarded, and sediment was suspended in 4 ml of distilled water. Subsequently, 1 mL of 0.67% thiobarbituric acid was added, and the mixture was heated in boiling water for 60 min. The mixture was then recentrifuged at 1600 g for 10 min. The absorbance of the organic layer was read at 532 nm using a spectrophotometer. Tetramethoxypropane was used as a standard, and MDA levels were calculated as nmol/ml.

Measurement of Total Oxidant Status

Serum TOS values were determined using a novel automated measurement method as previously described by Erel[14]. Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules, abundantly present in the reaction medium. The ferric ion made a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured by spectrophotomety, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide, and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L).

Measurement of Total Antioxidant Status

TAS values were determined using a novel-automated measurement method, developed by Erel[15]. In this method, hydroxyl radical (OH^-), which is the most potent biological radical, is produced. According to the manufacturer assay protocol, ferrous ion solution, present in Reagent 1 is mixed by hydrogen peroxide within Reagent 2. The sequentially produced radicals, such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, the antioxidative effect of the sample was measures against the potent free radical reactions, initiated by the hydroxyl radical produced. The assay has precision values lower than 3%. The results were expressed as mmol Trolox equivalent/liter (mmol Trolox Eq./L).

Calculation of Oxidative Stress Index

The TOS/TAS ratio was taken as the OSI. To perform the calculation, the units of TAS, mmol Trolox equivalent/L, were converted to µmol Trolox equivalent/L, and OSI was calculated using the formula OSI=[(TOS, µmol H_2O_2equivalent/L)/(TAS, µmol Trolox equivalent/L) x100][16].

DNA Extraction and JAK2 V617F Mutation Analyses

DNA was extracted using a MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostic, Penzberg, Germany) according to the manufacturer's instructions *(www.instructions.roche.com).* Genotyping was performed using a 7500 Real-Time PCR system [96 well format] (Applied Biosystem, Foster City, CA, USA) using a primer probe set of the JAK2 V617F system (Dr. Zeydanli Life Sciences, Ankara, Turkey) including a Tagman probe and with 5'-3' exonuclease activity. PCR reaction was performed according to the manufacturer's instructions. Briefly, the reactions were started at 95°C for 10 min, followed by 32 cycles of 95°C for 15 s and 60°C for 1 min.

Table I. Mean hematological values in ET patients and controls.

Hematologic	Patients (mean ± standard deviation)	Control group
Leukocyte (x103/µl)	11.9 ± 4.3	7.3 ± 1.9
Hemoglobin (g/dl)	13.4 ± 2.1	13.7 ± 1.4
Thrombocytes (x103/µl)	882 ± 315	224 ± 65
MPV(fl)	7.3 ± 0.76	8.6 ± 1
Neutrophils %	67 ± 10	57 ± 9.9
Lymphocytes %	20 ± 7.8	33 ± 9.5
Monocytes %	7 ± 3.3	6.1 ± 1.6
Eosinophils %	3.9 ± 4.4	2.8 ± 2
Basophils %	0.8 ± 0.6	0.6 ± 0.2

ET = essential thrombocythemia; MPV = mean platelet volume.

Statistical Analysis

Statistical data analysis was performed on SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Student's *t*-test was used to compare the patient and control groups and the paired *t*-test was used for within-group comparisons. Correlation analyses were performed using Spearman's correlation test. *p* value less than 0.05 was considered statistically significant.

Results

The patient and control group peripheral blood leukocyte and thrombocyte counts, hemoglobin and mean platelet volume (MPV) values and leukocyte formula are shown in Table I. Oxidative status parameters were not correlated with peripheral blood leukocyte counts, thrombocyte counts, hematocrit levels, MPV values, neutrophil, lymphocyte, monocyte, eosinophil and basophil percentages in the patient group (Table II). Furthermore, no correlations were noted between oxidative status parameters on the one hand and BMI, total cholesterol and low density lipoprotein levels (Table II). TOS, OSI and MDA were significantly higher, and TAS values significantly lower, in the patient group compared to the control group (Table III).

Differences between TAS, TOS, OSI and MDA levels in patients with or without diabetes mellitus, with or without hypertension, with high or normal total cholesterol levels and with high or normal BMI were not significant (Table IV).

A history of vascular event was present in 10 (23.2%) patients. Of these, 4 had cerebrovascular event (CVE), 5 coronary artery disease (CAD) and 1 deep venous thrombosis (DVT). TOS was higher in patients with a history of vascular event compared to those patients without. The other oxidative status parameters of these patients were not significantly different (Table V). Seven (70%) out of 10 patients with previous vascular event were also positive for JAK2V617F mutation.

After 6 months of treatment, the oxidative status parameters of 17 patients were measured. All of these patients received hydroxyurea and low-

Table II. Correlation analyses between oxidative stress parameters and hematologic parameters, BMI, total cholesterol and low density lipoprotein levels

	TAS		TOS		OSI		MDA	
Variables	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Leukocyte	0.21	0.17	0.03	0.82	-0.07	0.64	0.11	0.46
Hematocrit	-0.01	0.92	0.36	0.81	-0.005	0.97	0.11	0.44
Thrombocyte	0.17	0.25	-0.008	0.95	-0.11	0.46	-0.13	0.38
MPV	0.02	0.89	-0.02	0.88	0.01	0.91	0.25	0.10
Neutrophils	0.12	0.42	-0.08	0.60	-0.10	0.50	-0.18	0.26
Lymphocytes	-0.16	0.31	-0.06	0.71	0.001	0.99	0.10	0.50
Monocytes	-0.02	0.85	0.21	0.07	0.29	0.06	0.34	0.05
Eosinophils	0.02	0.87	0.02	0.90	-0.02	0.88	-0.06	0.71
Basophils	-0.07	0.65	0.22	0.17	0.23	0.13	0.19	0.22
BMI	0.03	0.88	0.12	0.53	0.12	0.54	0.36	0.06
CL	-0.22	0.06	-0.01	0.95	0.01	0.93	0.35	0.10
LDL levels	0.27	0.16	0.16	0.39	0.10	0.61	0.10	0.59

ET = essential thrombocythemia; MPV = mean platelet volume.

dose aspirin. OSI and MDA values were significantly decreased compared to the pre-treatment values. The post-treatment TAS values were increased compared to the pre-treatment values, although the difference was not statistically significant. Although post-treatment TOS values were markedly decreased compared to the pre-treatment values, no statistically significant difference was noted (Table VI).

JAK2V617F mutation positivity was determined in 28 (67.4%) of our patients. Oxidative status parameters of the patients with JAK2V617F mutation and without were similar (Table VII).

Discussion

Ischemic events in ET patients were frequently caused by arterial and less commonly by venous thrombosis. The significant factors in predicting thrombotic complications were age over 60 and a positive history of thrombosis[17-19]. Tendency to cardiovascular diseases[20,21], leukocytosis at time of diagnosis[22,23] and increased bone marrow fibrosis[24] constitute other risk factors. Bleeding tendency is common in these patients, and the risk increases when thrombocyte count is over 1500 $x10^3/\mu l$. Conditions including ischemia, hemorrhage, trauma, radioactivity and intoxication are some of the circumstances leading to oxidative stress. Oxidative stress is an indicator of increased intracellular reactive oxygen radicals. This condition leads to lipid peroxidation, and thus causes cellular damage and cell death[25]. Lipid peroxidation levels can be monitored by determining the levels of MDA or TOS, which are end products of lipid peroxidation[26]. TAS is produced from total protein (albumin representing 85%), uric acid, bilirubin, carotenoids, tocopherol and ascorbic acid; enzymes including glutathione peroxidase, catalase and superoxide dismutase play a role in the synthesis[27,28]. Oxidative stress, which can be described as a shift towards oxidant substances in the balance between oxidants and antioxidants, and has the potential to damage cellular structure, has been shown to be associated with various diseases in humans[29,30].

Oxidative status parameters such as TOS, OSI and MDA values were higher in our ET patients

Table III. Oxidative status parameters in patients and the control group

Parameters	Patients (n=43)	Control group (n=20)	*p* value
TAS (Mmol Trolox Eq./L)	1.36 ± 0.29	1.74 ± 0.36	<0.001
TOS (μmol H2O2 Eq./L)	19.8 ± 13.7	3.99 ± 1.00	<0.001
OSI (Arbitrary Unit)	1.51 ± 1.10	0.23 ± 0.06	<0.001
MDA (nmol/ml)	0.60 ± 0.07	0.07 ± 0.05	<0.001

TOS = total oxidative status; TAS = total antioxidant status; OSI = oxidative stress index; MDA = malondialdehyde.

Table IV. Oxidative status parameters versus DM, hypertension, total cholesterol and BMI.

	Diabetes Mellitus			Hypertension			Total Cholesterol			Body Mass Index		
	Present n=4	Absent n=39	p value	Present n=15	Absent n=28	p value	High n=15	Normal n=28	p value	High n=20	Normal n=23	p value
TAS (Mmol Trolox Eq./L)	1.43 ± 1.53	1.36 ± 0.30	0.65	1.48 ± 0.21	1.30 ± 0.31	0.05	1.39 ± 0.26	1.41 ± 0.23	0.82	1.44 ± 0.24	1.39 ± 0.26	0.58
TOS (µmol H2O2 Eq./L)	22.08 ± 12.40	20.15 ± 16.16	0.79	19.66 ± 15.19	20.69 ± 13.42	0.82	16.92 ± 12.76	22.66± 14.13	0.35	24.19 ± 15.5	18.45 ± 15.11	0.33
OSI (Arbitrary Unit)	1.52± 0.83	1.54±1.13	0.96	1.28 ± 0.93	1.68±1.18	0.26	1.32±1.16	1.61 ± 1.14	0.58	1.80 ± 1.32	1.27 ± 1	0.24
MDA (nmol/ml)	0.47 ± 0.27	0.62 ± 0.54	0.61	0.54 ± 0.55	0.63 ± 0.52	0.58	0.72 ± 0.63	0.51 ± 0.42	0.37	0.87 ± 0.71	0.52 ± 0.52	0.14

compared to the controls, whereas total antioxidant capacity, responsible for improving increased oxidative stress, was lower. In the 17 patients receiving cytoreductive treatment, OSI and MDA values decreased significantly following treatment. A history of vascular event was present in 10 patients. TOS values in these patients were higher than those in patients without a history of vascular event. Several studies have reported that antioxidant capacity, associated with the plasma levels of non-enzymatic antioxidants, is epidemiologically related to arteriosclerosis[31], ischemic diseases[32] and various types of cancers[33]. Vener et al's study of patients with primary myelofibrosis and post-polycythemia vera myelofibrosis demonstrated increased oxidative stress[34]. They also showed that this increase in oxidative stress was associated with a high level of homocysteine.

To the best of our knowledge, there are no published studies investigating oxidative stress status in ET patients. Our study was consistent with previous studies in myelofibrosis and other patient groups in terms of oxidative status indicators. TOS, OSI and MDA values, considered as oxidative stress indicators, were found higher, while TAS levels were lower compared to the control group. All these findings indicated increased oxidative stress in our patients. Furthermore, following treatment, MDA and OSI values were significantly lower compared to pretreatment. Oxidative stress was not correlated with hypertension, diabetes, triglyceride and cholesterol levels or BMI. This finding, and the presence of a significant decrease in oxidative stress parameters as a result of cytoreductive treatment, indicate that increased oxidative stress in ET patients was associated with the primary disease.

The pathological impact of oxidative stress in vascular disorders is well understood[11]. In one prospective study regarding ET patients, the cumulative prevalence of thrombotic complications within 27 months was reported as 24% in untreated, high-risk patients[2]. Consistent with published studies, we also determined a history of vascular event in 23.2% of our patients. In addition, most of the vascular events were also associated with the arterial system, in agreement with previous studies (5 CAD: coronary artery disease, 4 CVE: cerebrovascular events, 1 DVT: deep venous thrombosis). TOS values in patients with a history of vascular event were higher than those in patients without. However, there is still no satisfactory explanation why vascular event occurs in some ET patients and why other patients with similar biochemical and hematologic features do not ex-

Table V. The association between vascular event and oxidative status.

Parameters	With vascular event (n=10)	Without vascular event (n=33)	*p* value
TAS	1.52 ± 0.22	1.32 ± 0.30	0.06
TOS	28.42 ± 15.78	17.88 ± 12.50	0.03
OSI	1.92 ± 1.27	1.42 ± 1.04	0.21
MDA	0.80 ± 0.72	0.54 ± 0.44	0.17

TOS = total oxidative status; TAS = total antioxidant status; OSI = oxidative stress index; MDA = malondialdehyde.

Table VI. Patients' pre- and post-treatment oxidative status parameters.

Parameters	Pre-treatment (n=17)	Post-treatment (n=17)	*p* value
TAS	1.3 ± 0.37	1.5 ± 0.33	0.1
TOS	11.8 ± 9.9	6.9 ± 6.6	0.1
OSI	1.02 ± 0.9	0.44 ± 0.29	0.004
MDA	0.43 ± 0.28	0.23 ± 0.16	0.034

TOS = total oxidative status; TAS = total antioxidant status; OSI = oxidative stress index; MDA = malondialdehyde.

Table VII. Oxidative status parameters in JAK2V617F mutation positive and negative ET patients.

Parameters	JAK2V617F Positive (n=28)	JAK2V617F Negative (n=15)	*p* value
TAS	1.38 ± 0.25	1.33 ± 0.37	0.64
TOS	21.97 ± 14.23	17.27 ± 13.14	0.29
OSI	1.62 ± 1.13	1.39 ± 1.06	0.52
MDA	0.62 ± 0.50	0.57 ± 0.58	0.79

TOS = total oxidative status; TAS = total antioxidant status; OSI = oxidative stress index; MDA = malondialdehyde.

perience it. The findings of this study demonstrate that increased oxidative stress may be associated with an increased risk of vascular event. Even though the causes of increased oxidative stress in ET patients are not yet fully understood, a rise in homocysteine levels, as in myelofibrosis patients, or endothelial damage caused by ischemic changes may be responsible for the pathogenesis.

Conclusions

Our findings indicate that oxidative stress parameters in ET patients were significantly increased, while antioxidant capacity, which was expected to correct the situation, was significantly decreased compared to the healthy individuals. Furthermore, TOS values in patients with previous vascular events were significantly higher than in patients without vascular events. This finding indicates that oxidative stress may be associated with vascular events. Determining oxidative status parameters would be helpful in the prevention, early diagnosis and treatment of vascular events. Large, well-designed studies are now needed to assess whether or not oxidative status parameters can be indicators of vascular events.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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