

The Thrombotic Events in Polycythemia Vera Patients May Be Related to Increased Oxidative Stress

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Key Words

Polycythemia vera · Oxidative stress · Total antioxidant status · Vascular events · JAK2V617F mutation

Abstract

Objective: This study was designed to compare the oxidative stress parameters of patients with polycythemia vera (PV) to those of healthy volunteers and to investigate the probable relationship between vascular events and parameters of oxidative status such as total oxidative status (TOS), total antioxidant status, oxidative stress index (OSI) and malondialdehyde (MDA) in PV patients. **Material and Methods:** Thirty-five PV patients (20 males and 15 females) and 20 healthy volunteers (11 males and 9 females) were enrolled. The oxidative status parameters of the patients were measured by spectrophotometric analyses at the time of diagnosis and at 6 months after treatment which consisted of phlebotomy and 100 mg/day acetyl salicylic acid with or without hydroxyurea for the high- and low-risk disease group, respectively. These parameters were compared both to healthy controls and to each other, in order to obtain the values before and after treatment. In addition, during diagnosis, the

oxidative status parameters of patients with PV and a history of a vascular event were compared with those of patients with no history of a vascular event. **Results:** The TOS, OSI and MDA values were significantly higher in the patients than in the control group at the time of diagnosis. At 6 months after phlebotomy and 100 mg/day acetyl salicylic acid therapy, the TOS, OSI and MDA values were significantly lower in the patients when compared to the pretreatment values. The TOS and OSI levels were notably higher in the patients with a vascular-event history than in those without this history. **Conclusion:** Oxidative stress parameters were increased in PV patients.

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Introduction

Polycythemia vera (PV) is a chronic, progressive, clonal, myeloproliferative disease with an insidious onset characterized by an absolute increase in red-cell mass, leukocytosis, thrombocytosis and splenomegaly. The annual incidence of PV is between 2.3 and 2.8 in 100,000 patients. Vascular thrombosis is the most frequent cause

of death in PV patients [1]. This is believed to be a direct consequence of the increased red-cell mass and hyperviscosity. However, the thrombotic tendency in PV may be multifactorial due to the involvement of the abnormal number of platelets and leukocytes [2]. Increased blood viscosity can also result in bleeding disorders, and these ischemic vascular events may be seen at diagnosis or during the follow-up of PV patients, with or without treatment. However, there are no standard patient characteristic criteria to predict the development of vascular events.

Oxidative stress, detected in many diseases, is associated with disruption of the pro-oxidant and antioxidant balance, in favor of the former [3]. Development of cells in an oxygen-containing environment is problematic without a strong defense system, known as the antioxidant system, which includes enzymatic and nonenzymatic components. An increased oxidative state has been reported in diseases such as diabetes mellitus [4] and chronic renal failure [5]. In response to increased oxidative stress, living organisms develop an antioxidant defense to prevent the harmful effects of free oxygen radicals. Individual measurement of all the different antioxidants is difficult due to the workload involved in the laboratory, the high costs incurred, the complex techniques required and the interaction of the serum antioxidants [6].

New methods for measuring all these antioxidants expressed as a single value, i.e. serum total antioxidant status (TAS), have been developed [6]. The TAS is more easily measured at less cost and in a very short time [6]. Oxidative stress has been shown to be an important contributor to endothelial dysfunction and atherosclerosis [7]. The pathological role of oxidative stress in vascular diseases has been well described [8]. Hence, this technique was used to investigate the status of oxidative stress in PV patients, based on the potential association between increased vascular events in these patients and their oxidative state.

Materials and Methods

After giving informed consent, 35 PV patients, diagnosed according to the 2008 criteria of the World Health Organization [9], and 20 healthy volunteers with similar demographic characteristics were enrolled in the study, conducted from January 2009 until June 2011 at the Hematology Clinic, Kanuni Training and Research Hospital, Trabzon, Turkey. Local Ethics Committee approval was granted, and the study was carried out in accordance with the ethical standards specified in the 1964 Declaration of Helsinki.

The study group consisted of 20 male and 15 female patients with an age range of 38–84 years; the age range of the control group of 11 males and 9 females was 34–80 years.

Table 1. Clinical and demographic characteristics of the PV patients and the control group

	Patients, n	Control group, n
Total number	35	20
Median age (range), years	60.7 (38–84)	57.6 (34–81)
Male/female	20/15	11/9
Splenomegaly	7	–
Itching	10	–

Exclusion criteria were a history of alcohol consumption, intravenous drug abuse, pregnancy, consumption of antioxidants such as vitamin E, β -carotene, ascorbic acid, glutathione, probucol, fish oil or ferrous sulfate consumption and having a condition such as HIV infection, rheumatoid arthritis, cirrhosis, acute infection or a malignant disease.

Risk Groups and Treatment

Bone marrow aspiration and biopsy were performed on the patients in the study, in addition to the analysis of hematological and biochemical parameters. Patients were divided into 2 groups: a low-risk group (age <60 years with no history of thrombosis) consisting of 15 patients and a high-risk group (age $\geq$ 60 years or a history of thrombosis) consisting of 20 patients [10]. The low-risk patients were treated with phlebotomy and low-dose acetyl salicylic acid (100 mg/day); patients at a high risk were in addition treated with hydroxyurea.

For the investigation of oxidative stress parameters, 10-ml peripheral venous blood samples were collected from patients at the time of diagnosis and at the end of 6 months of treatment. Blood samples for oxidative stress parameters were also collected from the control group. Blood samples were centrifuged at 3,000 rpm for 10 min and the resulting serum samples were stored in a deep-freezer (-30°C) until the assay for serum oxidative stress status markers was performed. Serum samples were subsequently removed from the freezer and thawed before conducting the spectrophotometric measurements (as defined below), and were then analyzed for the TAS, total oxidative status (TOS), malondialdehyde (MDA) and oxidative stress index (OSI) values.

Lipid peroxidation in human serum samples was determined as MDA concentration (nmol/ml) using the method described by Yagi [11]. TOS was determined using a novel automated measurement method as previously described by Erel [12]. Serum TOS levels were calculated in $\mu\text{mol H}_2\text{O}_2$ Eq/l.

TAS was determined using a novel automated measurement method developed by Erel [13]. Serum TAS levels were calculated in mmol Trolox Eq/l. OSI was calculated by first converting the units of TAS, mmol Trolox Eq/l to $\mu\text{mol Trolox Eq/l}$, and using the formula $\text{OSI} = [(\text{TOS}, \mu\text{mol H}_2\text{O}_2 \text{ Eq/l}) / (\text{TAS}, \mu\text{mol Trolox Eq/l}) \times 100]$ [14]. DNA extraction was performed by extracting DNA using a MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Penzberg, Germany) according to the manufacturer's instructions (www.instructions.roche.com). The genotyping of JAK2V617F mutation was performed using a 7500 Real-Time PCR system (96-well format; Applied Biosystems, Foster City, Calif., USA) us-

ing a primer probe set of the JAK2V617F system (Dr. Zeydanli Life Sciences, Ankara, Turkey) including a TaqMan probe with 5'-3' exonuclease activity. The PCR reaction was set according to the manufacturer's instructions. Briefly, reactions were started at 95°C for 10 min, and followed by 32 cycles at 95°C for 15 s and 60°C for 1 min.

Statistical Analysis

The Kolmogorov-Smirnov test was used to determine whether or not the distribution was normal ($p > 0.05$). Statistical analysis of the data was performed using the SPSS 17.0 software package. The Student *t* test was used to compare the patient and control groups and the paired *t* test was used to perform within-group comparison analyses. Correlation analyses were performed using the Spearman correlation test. $p < 0.05$ was accepted as statistically significant.

Results

Demographic and clinical characteristics of the patient and control groups are shown in table 1. TOS, OSI and MDA values were significantly higher in the patient group, but not the TAS values (table 2).

The bone marrow aspiration biopsy of the patients revealed hypercellularity and findings of hyperplasia in all three cellular series (erythroid, granulocytic and megakaryocytic). Hematological findings from the patients and healthy controls are shown in table 3. Within the PV group, there was no statistically significant ($p > 0.05$) difference between patients with and without conditions affecting oxidative stress parameters such as diabetes mellitus (*p* values for TAS, TOS and OSI were 0.15, 0.14 and 0.16, respectively), hypertension (*p* values for TAS, TOS and OSI were 0.47, 0.51 and 0.33, respectively), obesity (*p* values for TAS, TOS and OSI were 0.43, 0.37 and 0.4, respectively), hyperlipidemia (*p* values for TAS, TOS and OSI were 0.77, 0.95 and 0.94, respectively) and smoking (*p* values for TAS, TOS and OSI were 0.73, 0.37 and 0.4, respectively).

No correlation was determined between oxidative stress parameters, hemoglobin levels, leukocyte counts, thrombocyte counts, total cholesterol levels, low-density lipoprotein cholesterol levels and BMI values. After 6 months of treatment, the oxidative parameters of the patients were measured again. TOS, OSI and MDA levels were significantly decreased in treated patients compared to pretreatment values (TOS values before and after treatment were 14.8 ± 10 and 6.8 ± 6.1 , respectively; $p = 0.019$; OSI values before and after treatment were 0.92 ± 0.63 and 0.41 ± 0.32 , respectively; $p = 0.011$; MDA values before and after treatment were 0.55 ± 0.52 and 0.25 ± 0.40 ,

Table 2. Oxidative status parameters in the PV patients and the control group

Oxidative status parameters	Patients (n = 35)	Control group (n = 20)	p value ^a
TOS, $\mu\text{mol H}_2\text{O}_2$ Eq/l	17.3 ± 13.8	3.90 ± 1.00	<0.001
TAS, mmol Trolox Eq/l	1.63 ± 0.32	1.74 ± 0.36	NS
OSI, arbitrary units	1.05 ± 0.84	0.23 ± 0.06	<0.001
MDA, nmol/ml	0.42 ± 0.40	0.07 ± 0.05	<0.001

Values are expressed as mean $\pm$ SD. NS = Nonsignificant.

^a For comparison between control and study population.

Table 3. Mean hematological values in the PV patients and the control group

Parameters	Patients (n = 35)	Control group (n = 20)
WBC, $\times 10^3/\mu\text{l}$	11.08 ± 3.97	7.34 ± 1.90
Hemoglobin, g/dl	18.4 ± 2.1	13.7 ± 1.4
Platelet count, $\times 10^3/\mu\text{l}$	424 ± 265	224 ± 65
MPV, fl	8.1 ± 0.9	8.6 ± 1
Neutrophils, %	71 ± 8.3	57 ± 9.9
Lymphocytes, %	20 ± 7.1	33 ± 9.5
Monocytes, %	5.4 ± 2.8	6.1 ± 1.6
Eosinophils, %	1.7 ± 1.0	2.8 ± 2
Basophils, %	0.9 ± 0.9	0.6 ± 0.2

Values are expressed as mean $\pm$ SD. MPV = Mean platelet volume; WBC = white blood cell count.

Table 4. Relationships between vascular events and oxidative status in the PV patients

Oxidative parameters	Vascular event		p value ^a
	positive (n = 10)	negative (n = 25)	
TOS, $\mu\text{mol H}_2\text{O}_2$ Eq/l	26.72 ± 16.52	12.46 ± 9.17	0.03
TAS, mmol Trolox Eq/l	1.57 ± 0.24	1.65 ± 0.35	NS
OSI, arbitrary units	1.64 ± 1.01	0.76 ± 0.56	0.02
MDA, nmol/ml	0.48 ± 0.37	0.41 ± 0.41	NS

Values are expressed as mean $\pm$ SD. NS = Nonsignificant.

^a For comparison between PV patients with positive and those with negative vascular events.

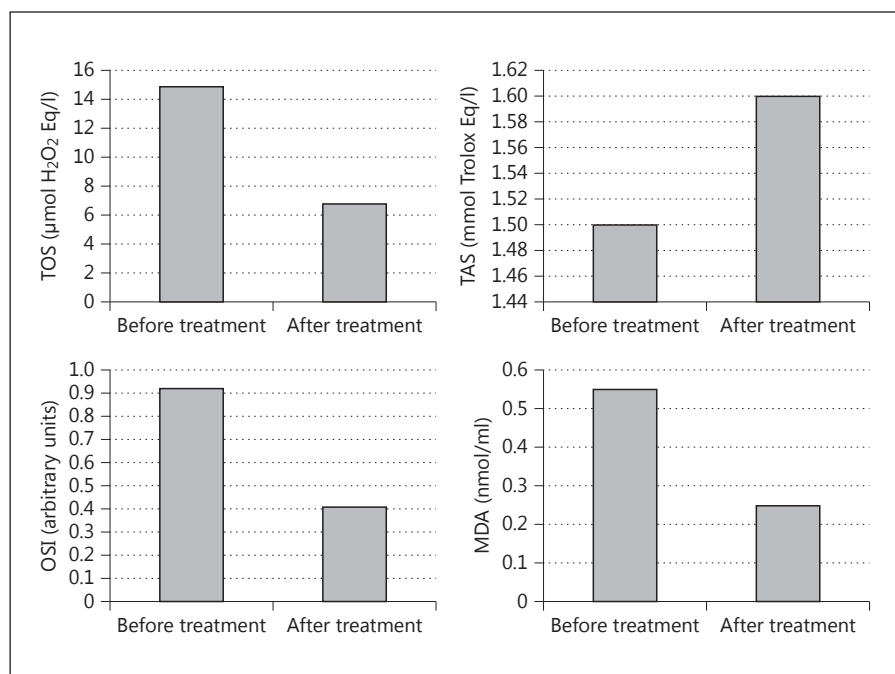


Fig. 1. Oxidative status parameters of patients with PV before and after treatment.

respectively; $p = 0.027$). There was no statistically significant difference between the TAS values before and after treatment ($p > 0.05$; fig. 1).

Twenty-eight of the 35 PV patients were found to be positive for JAK2V617F mutation. The comparison of oxidative stress parameters between the PV patients who were positive and negative for JAK2V617F revealed no significant difference. Ten (28.5%) of the 35 patients had a history of vascular events (7 had coronary artery disease, 1 had portal-vein thrombosis, 1 had a cerebrovascular event and 1 had deep-vein thrombosis). TOS and OSI values were notably higher in patients with a history of vascular events compared to patients without this history. In contrast, TAS and MDA levels were similar in both groups (table 4). In addition, of the 10 patients with a history of vascular events, 8 were positive for JAK2V617F mutation.

Discussion

Vascular events were the major inadvertent complications in PV. To establish whether or not increased oxidative stress might be an underlying cause of such events in PV patients was the main objective of our study. The levels of TAS, TOS, OSI and MDA were the known parameters for evaluating the antioxidant status of patients. We

examined these levels in PV patients and found that all of them, except for the TAS values, were significantly higher than those in the control group. After 6 months of treatment, the TOS, OSI and MDA levels were significantly decreased in treated patients compared to before treatment.

In a recent study, the MDA levels of 35 lung cancer patients were significantly higher than those in the control group [15]. Vener et al. [16] also reported increased oxidative stress in patients with primary myelofibrosis and PV-associated myelofibrosis. They determined that the increase in oxidative stress was linked to increased homocysteine levels. We, for the first time, have shown increased oxidative stress in essential thrombocythemia (ET) patients by referring to the TAS, TOS, OSI and MDA values. We also reported that oxidative stress parameters were decreased after treatment in ET [17]. Ischemia, hemorrhage, trauma, radioactivity and intoxications are also considered to be causative conditions for oxidative stress [18]. We studied oxidative stress parameters (TOS, OSI and MDA) in PV patients and found them to be significantly higher than in the control group. In addition, after treatment of the PV patients with phlebotomy and low-dose acetyl salicylic acid (100 mg/day), the TOS, OSI and MDA values had decreased significantly when compared to the pretreatment values. The absence of a relationship between increased oxidative stress and hyper-

tension, diabetes, smoking, hyperlipidemia and BMI as well as the remarkable decrease observed in the oxidative stress parameters of these patients after treatment suggests that the rise in oxidative stress was indeed related to PV. Although we did not fully understand the reason for the increased oxidative stress in our patients, in the light of the literature, we suggest that it may have been associated with ischemic changes due to endothelial damage caused by a rise in the level of homocysteine.

An advanced age (≥ 60 years) and a history of thrombosis are the two most important prognostic factors for the development of vascular complications in PV patients. In some studies, the possibility of a thrombotic event developing over a 10-year period has been reported to be at least 40–60% in these patients. It has been reported that oxidative stress is well known to play a crucial pathological role in vascular diseases [8]. The finding of 28.5% of patients with a history of thrombosis is consistent with the previous study of Wehmeler et al. [19], in which thrombotic episodes were observed in one third and bleeding in one quarter of the patients in the series. In our previous study on ET, we found the TOS levels to be higher in patients with vascular events than in those without them [17]. In this study, a history of vascular events was present in 10 patients (23.2%): 4 had cerebrovascular events, 5 had coronary artery disease and 1 had deep-vein thrombosis. Similarly, in this study, 10 PV patients (28.5%) had a history of previous vascular events, and their TOS and OSI values were significantly higher when compared to those without such events. The reason why vascular events occur in some PV patients has not yet been clearly elucidated, though the 2 groups in our study had values that were similar. Our findings suggest that increased oxidative stress in PV patients may be associated with an increased risk of vascular events.

JAK2V617F mutation has been shown to play a major role in the pathogenesis of myeloproliferative diseases, particularly PV, ET and primary myelofibrosis. However, a few studies suggest that JAK2V617F mutation may not be the initiator of the disease [20]. It is considered to be a late genetic event occurring in patients with myeloproliferative disease, as reported in our recent studies [21–23]. The role played by free radicals in causing DNA damage and the pathways of cellular oxidative damage in neoplastic transformation have been well defined [24]. In this study, oxidative stress parameters were similar in patients who were negative and positive for the JAK2V617F mutation as well as in those with ET [17]. As 80% of our thrombotic patients were positive for the JAK2V617F mutation, increased oxidative stress by itself could be the cause of the mutagenicity which led to JAK2V617F mutation. In other words, we suggest that JAK2V617F-negative patients might become positive under the effects of increased oxidative stress.

Conclusion

In this study, the markers of oxidative stress (TOS, OSI and MDA) in PV patients were significantly higher than those in the control group, with no change in antioxidant capacity. In addition, TOS and OSI values in patients with a history of vascular events were significantly higher than in those without this history. This finding suggests that increased oxidative stress may be related to vascular events in addition to mutagenesis such as JAK2V617F mutation in PV patients. Oxidative parameters could be used as an indicator for the prevention, recognition and early treatment of vascular events. Further comprehensive studies are now needed to confirm this.

References

- Chievitz E, Thiede T: Complications and causes of death in polycythemia vera. *J Intern Med* 1962;2:513–523.
- Falanga A, Marchetti M, Evangelista V, et al: Polymorphonuclear leukocyte activation and hemostasis in patients with essential thrombocythemia and polycythemia vera. *Blood* 2000;96:4261–4266.
- Sies H: Oxidative stress: introductory remarks; in Sies H (ed): *Oxidative Stress*. London, Academic Press, 1985, pp 1–8.
- Mezzetti A, Cipollone F, Cuccurullo F: Oxidative stress and cardiovascular complications in diabetes: isoprostanes as new markers on an old paradigm. *Cardiovasc Res* 2000;47:475–488.
- Horoz M, Aslan M, Koylu AO, et al: The relationship between leptin level and oxidative status parameters in hemodialysis patients. *Artif Organs* 2009;33:81–88.
- Erel O: A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 2004;37:277–285.
- Tepel M: Oxidative stress: does it play a role in the genesis of essential hypertension and hypertension of uraemia? *Nephrol Dial Transplant* 2003;18:1439–1442.
- Madamanchi NR, Vendrow A, Runge MS: Oxidative stress and vascular diseases. *Arterioscler Thromb Vasc Biol* 2005;25:29–38.
- Swerdlow SH, Campo E, Harris NL, et al: *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, International Agency for Research on Cancer, 2008, pp 180–182.

- 10 Cervantes F, Passamonti F, Barosi G: Life expectancy and prognostic factors in the classic BCR-ABL negative myeloproliferative disorders. *Leukemia* 2008;22:905–914.
- 11 Yagi K: Lipid peroxides and related radicals in clinical medicine; in Armstrong D (ed): *Free Radicals in Diagnostic Medicine*. New York, Plenum Press, 1994, pp 1–15.
- 12 Erel O: A new automated colorimetric method for measuring total oxidant status. *Clin Biochem* 2005;38:1103–1111.
- 13 Erel O: A novel automated method to measure total antioxidant response against potent free radical reactions. *Clin Biochem* 2004;37: 112–119.
- 14 Aycicek A, Erel O, Kocyigit A: Decreased total antioxidant capacity and increased oxidative stress in passive smoker infants and their mothers. *Pediatr Int* 2005;47:635–639.
- 15 Özkurt S, Demir S, Köseoğlu MH, et al: Plasma malondialdehyde levels and sulfhydryl content in patients with lung cancer. *Solum* 2000;2:96–99.
- 16 Vener C, Novembrino C, Catena FB, et al: Oxidative stress is increased in primary and post-polycythemia vera myelofibrosis. *Exp Hematol* 2010;38:1058–1065.
- 17 Durmus A, Mentese A, Yilmaz M, et al: Increased oxidative stress in patients with essential thrombocythemia. *Eur Rev Med Pharmacol Sci* 2013;17:2860–2866.
- 18 Cross CE, Halliwell B, Borish ET, et al: Oxygen radicals and human disease. *Ann Intern Med* 1987;107:526–545.
- 19 Wehmeler A, Daum I, Jamin H: Incidence and clinical risk factors for bleeding and thrombotic complications in myeloproliferative disorders. A retrospective analysis of 260 patients. *Ann Hematol* 1991;63:101–106.
- 20 Philip AB, Anthony RG: Essential thrombocythemia; in Kenneth K, Marshall AL, Ernest B, Thomas JK, Uri S, Josef TP (eds): *Williams Hematology*, vol 87, ed 8. Springfield, McGraw Hill, 2010, pp 1237–1245.
- 21 Akalin I, Armangil D, Durmus A: Neonatal polycythemia may be free of JAK2V617F mutation. *Türkiye Klinikleri J Med Sci* 2013; 33:132–137.
- 22 Kralovics R, Teo SS, Buser AS, et al: Altered gene expression in myeloproliferative disorders correlates with activation of signaling by the V617F mutation of Jak-2. *Blood* 2005;106: 3374.
- 23 Olcaydu D, Harutyunyan A, Jäger R, et al: A common Jak-2 haplotype confers susceptibility to myeloproliferative neoplasms. *Nat Genet* 2009;41:450.
- 24 Aliyazicioglu Y, Demir S, Turan I, et al: Preventive and protective effects of Turkish propolis on H₂O₂-induced DNA damage in foreskin fibroblast cell lines. *Acta Biol Hung* 2011;62:388–396.

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