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ORIGINAL PAPER

THE EVALUATION OF OXIDATIVE STRESS IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA TREATED WITH RISK-ADAPTED THERAPY

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Abstract

Introduction. Essential thrombocythemia (ET) is a clonal disorder of the hematopoietic stem cells characterized by persistent thrombocytosis in the peripheral blood, excessive proliferation of megakaryocytes and minor reticulin fibrosis in the bone marrow. It seems that oxidative stress is involved in the development and progression of ET.
Objective. To evaluate oxidative stress levels in ET patients treated with risk-adapted therapy. **Material and methods.** 62 ET patients and 20 controls (informed consent obtained) were enrolled. ET diagnosis was based on WHO criteria (2016 revised). Reactive oxygen species (ROS) levels and the total antioxidant capacity (TAC) were evaluated at time of diagnosis and after 6 months of risk-adapted therapy. ET patients were divided into 3 groups and treated with risk–adapted therapy: a low risk group, treated with low doses of aspirin 75 mg/day or watch-and-wait; an

Résumé

Évaluation du stress oxydatif chez les patients atteints de thrombocytémie essentielle sous traitement adapté au risque

Introduction. La thrombocytémie essentielle (TE) est une maladie de la cellule-souche hématopoïétique caractérisée par une thrombocytose persistante dans le sang périphérique, une prolifération excessive des mégacaryocytes et une faible fibrose réticulinique dans la moelle osseuse. Il semble que le stress oxydatif soit impliqué dans le développement et la progression de la TE.
L'objectif de l'étude est d'évaluer le niveau du stress oxydatif chez les patients atteints de TE sous traitement adapté au risque.
Materiel et méthodes. 62 patients atteints de TE et 20 volontaires sains (consentement écrit obtenu) ont été inscrits. Le diagnostic a été fait second les critères OMS 2016 pour la TE (version révisée en 2016). Le

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intermediate risk group treated with low doses of aspirin 100 mg/day or low-dose aspirin + cytoreductive treatment; a high-risk group, treated with low doses of aspirin and cytoreductive treatment (hydroxyurea) or platelet-lowering agents (anagrelide).

Results. ET patients had at diagnosis higher ROS levels and a lower TAC vs. controls. After 6 months of risk-adapted therapy, ROS levels decreased and TAC increased. No significant differences were seen between the effect of hydroxyurea and the effect of anagrelide on oxidative stress levels.

Conclusions. ROS levels are increased and TAC is decreased in ET patients vs. controls. These values depend on the risk group assigned to the patient. Risk-adapted therapy was useful to reduce ROS levels and increase TAC.

Keywords: essential thrombocythemia, oxidative stress, reactive oxygen species, total antioxidant capacity, risk-adapted therapy.

niveau d'espèces réactives de l'oxygène (ERO) et la capacité antioxydante totale (CAT) ont été mesurés au moment du diagnostic et 6 mois après le commencement du traitement adapté au risque. Les patients avec TE ont été divisés en 3 groupes et ont reçu un traitement adapté au risque: groupe dit de «faible risque», traité avec une faible dose d'aspirine 75 mg/jour ou watch-and-wait; groupe dit de «risque intermédiaire», traité avec une faible dose d'aspirine 100 mg/jour ou une faible dose d'aspirine + traitement cytoréducteur; groupe dit de «haut risque», traité avec une faible dose d'aspirine et traitement cytoréducteur ou plaquettes-réducteur (anagrelide).

Résultats. Les patients avec TE ont eu au moment du diagnostic un taux augmenté des ERO et une CAT moindre comparés aux témoins. Après 6 mois du traitement adapté au risque, le taux des ERO a subi une baisse et la CAT une augmentation. Nous n'avons pas trouvé une différence statistiquement significative entre l'effet de l'hydroxyurée ou l'effet de l'anagrelide sur le niveau du stress oxydatif.

Conclusions. Le taux des ERO est augmenté et la CAT est diminuée chez les patients atteints de TE vs. groupe témoins. Ces taux dépendent des groupes à risque assignés aux patients. Le traitement adapté au risque a été efficace pour abaisser le taux des ERO et augmenter la CAT.

Mots-clés: thrombocytémie essentielle, stress oxydant, espèces réactives de l'oxygène, capacité antioxydante totale, traitement adapté au risque.

Introduction

Essential thrombocythemia (ET) is a clonal disorder of the hematopoietic stem cells, characterized by persistent thrombocytosis in the peripheral blood, excessive proliferation of megakaryocytes in the bone marrow, normal red cell mass and absence of prominent bone marrow fibrosis. In ET, gene expression profiles in megakaryocytes are altered: there is a decreased expression of pro-apoptotic genes, whereas the expression of anti-apoptotic genes evolves oppositely[1,2]. An estimate of 50% of acquired ET cases test positive for a mutation in exon 14 of the JAK2 gene (JAK2V617F – a point mutation in codon 617 leads to the substitution of valine with phenylalanine). The JAK2V617F mutation is involved in the rise in platelet numbers, thrombocytes being hypersensitive to cytokine stimulation[3]. In 35% of cases, mutations can occur in the exon 12 of the JAK2 gene, in the calreticulin gene (CALR) or in the MPL gene, whereas 15% of patients are triple negative for JAK2, CALR or MPL mutations[4]. In ET, the increased count of immature platelets is associated with a higher rate of thrombosis independently of thrombocytosis[5]. Moreover, the JAK2V617F mutation is associated with a higher number of immature platelets in ET[6].

A patient is diagnosed with ET if all four major World Health Organization (WHO) criteria are met: 1. persistent thrombocytosis >450.000/mmc in the peripheral blood; 2. bone marrow megakaryocyte proliferation with large megakaryocytes of mature morphology, hyperlobulated nuclei and minor reticulin fibrosis at the histological examination of the bone marrow; 3. exclusion of chronic myeloid leukemia, polycythemia vera, primary myelofibrosis, myelodysplastic syndromes or other myeloid neoplasms; 4. detection of JAK2, CALR or MPL mutations. In some cases, ET diagnosis can be established if the first three major criteria and one minor criterion are met. The minor criterion can be the presence of another clonal marker or the exclusion of reactive thrombocytosis. Oxidative stress has a major role in carcinogenesis and disease progression in myeloproliferative neoplasms, via increased levels of reactive oxygen

species (ROS). ROS activate proinflammatory pathways (NF-kB, NF-E2) and induce a low-grade chronic inflammation, key factors in genomic instability and progression to myelofibrosis or leukemic transformation[7-11].

ET treatment is risk-adapted: a) very low risk patients do not require any treatment; b) low risk cases are treated with low doses of aspirin 75-100 mg/day; c) intermediate risk ET requires aspirin in low doses (100 mg/day) ± cytoreductive treatment; d) high risk patients require low doses of aspirin + cytoreductive treatment or platelet-reducing therapy[4,12].

The Objective of our study was to evaluate the levels of oxidative stress in ET patients and to study the influence of risk-adapted therapy on oxidative stress levels.

Materials and Methods

The study group consisted in 62 ET patients admitted to the Hematology Clinic of Filantropia City Hospital of Craiova and to the Department of Hematology, County Emergency Hospital Slatina, Romania. The control group consisted in 20 healthy volunteers with similar demographic characteristics. All procedures were carried out in accordance with the ethical standards specified in the Declaration of Helsinki and had the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova (approval number: 79/23.02.2017). The patients were diagnosed with ET based on the WHO criteria (2016 revised)[4]. Hematological and biochemical parameters, such as acute phase proteins (fibrinogen, CRP), iron parameters (serum iron, transferrin saturation, ferritin), glycemia, total cholesterol levels, HDL-cholesterol, LDL-cholesterol, triglycerides, were analyzed. Bone marrow aspiration/biopsy was performed. ECG, echocardiography and ultrasound scans of the upper abdomen with measurement of spleen size were also employed. Secondary causes of thrombocytosis, such as iron deficiency associated with chronic blood loss, chronic inflammatory diseases, chronic infections, malignancies, were ruled out.

Patients were divided by sex, age, history of vascular events, platelet count and presence of JAK2V617F/CALR/MPL mutations. Based on age, platelet count and history of thrombosis, ET patients were divided into 3 groups and treated with risk-adapted therapy: a low risk group (age < 60 years, asymptomatic, without cardiovascular risk factors, platelet count = 400-1500 x 10^3 platelets/μL) treated with low doses of aspirin (75 mg/day) or watch-and-wait, an intermediate risk group (age 40-60 years, with microvascular occlusions, without cardiovascular risk factors or thrombosis, platelets = 400-1500 x 10^3 platelets/μL) treated with low-dose aspirin ± cytoreductive treatment, and a high risk group (age > 60 years, presence of vascular risk factors, history of thrombosis/hemorrhage, platelet count > 1500 x 10^3 platelets/μL) treated with low-dose aspirin and cytoreductive treatment (hydroxyurea) or platelet-lowering therapy (anagrelide).

Oxidative stress was evaluated by flow cytometry (CyFlow Space Sysmex, Abcam detection kit) to quantify ROS values and using a multidetection microplate reader (FLUOstar Omega, reagents from Sigma-Aldrich) to measure the total antioxidant capacity (TAC). Positive and negative control samples were prepared according to the manufacturer's instructions. All measurements were performed at the time of diagnosis and 6 months after the initiation of risk-adapted therapy. Results were compared both to healthy controls and in between ET patients before and after risk-adapted therapy. Statistical analysis of data was performed and a p-value ≤ 0.05 was considered statistically significant.

Results

The study group included 62 ET patients: 37 women (59.68%) and 25 men (40.32%). The mean age of the patients was 59.50 years and the age range was 22-82 years. Most of the patients were in the 7th life decade. The mean hematological parameters of the study and control groups are depicted in **Table 1**. Data is presented as mean value ± standard deviation.

ROS values were higher and TAC was lower in men vs. women. JAK2V617F mutation was detected in approximately 60% of ET patients. ROS values were higher (2.73 mM/L vs. 2.60 mM/L)

Table 1. Hematological parameters in ET patients vs. controls.

Hematological parameter	*ET patients*	*Controls*
Hemoglobin (g/dL)	11.5 ± 2.3	12.7 ± 1.2
Leukocytes (x10^3 leukocytes/μL)	12.31 ± 4.1	6.8 ± 1.7
Neutrophils (%)	61 ± 9	58 ± 8
Eosinophils (%)	3.1 ± 1.2	2.5 ± 1
Basophils (%)	0.6 ± 0.5	0.5 ± 0.2
Lymphocytes (%)	22 ± 6	28 ± 8
Monocytes (%)	8 ± 2.1	5.6 ± 1.4
Platelets (x10^3 platelets/μL)	793 ± 324	272 ± 84
Mean Platelet Volume (fL)	7.4 ± 0.6	8.4 ± 1.1

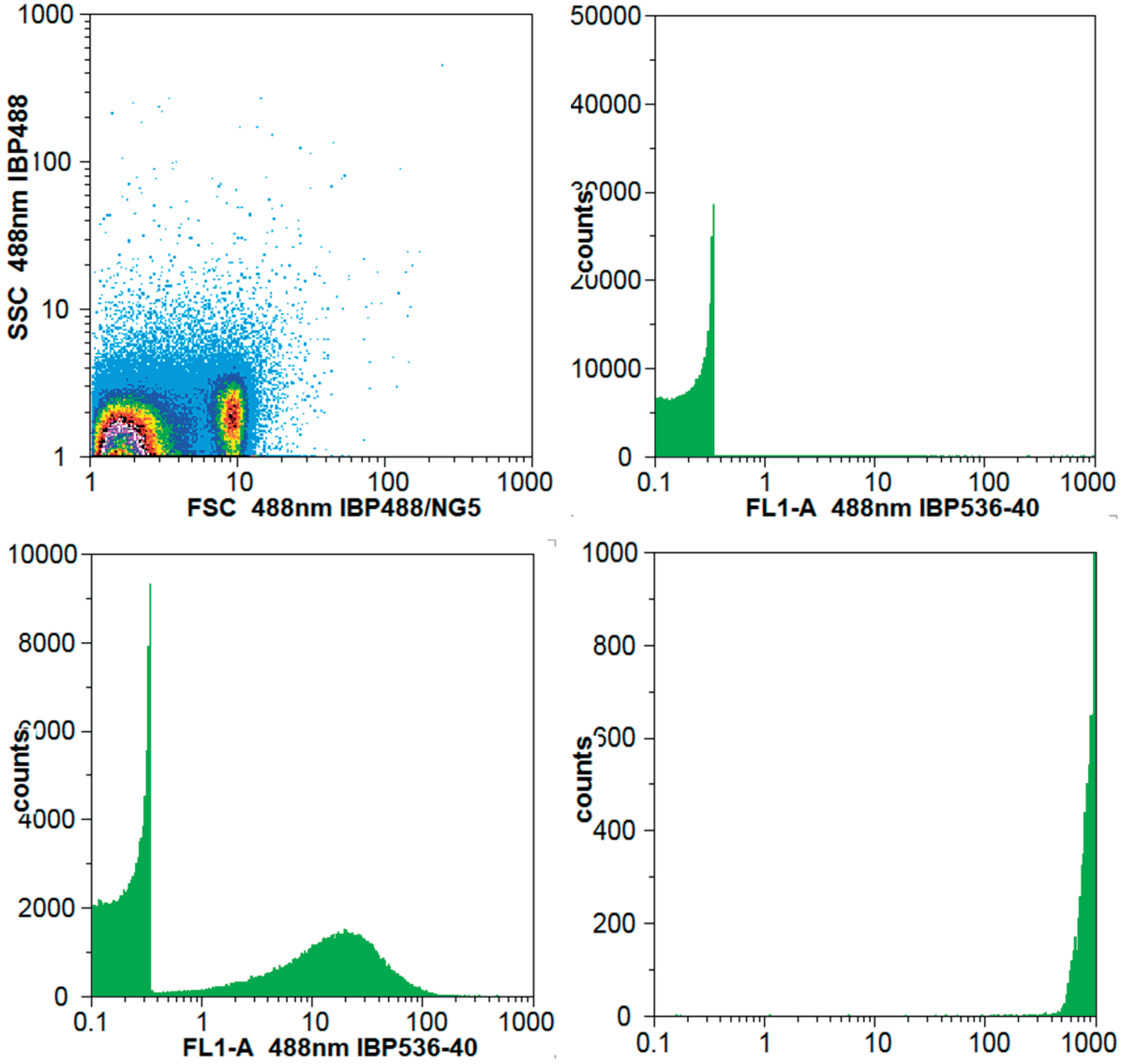

Figure 1. Fluorescence evaluation by flow cytometry in a 56-year-old ET patient vs. positive and negative controls.

and TAC was lower (0.46 mM/L vs. 0.47 mM/L) in JAK2V617F-positive vs. JAK2V617F-negative patients. The difference between the two groups was statistically significant ($p < 0.05$). Figure 1 depicts the results of fluorescence evaluation by flow-cytometry in a patient with ET vs. positive and negative controls.

Arterial hypertension and coronary heart disease were the most frequent comorbidities in the study group (approximately 50% of ET patients), followed by type 2 diabetes mellitus and obesity (approximately 20% of ET patients). Vascular complications were found in 14 patients (22.5%): 3 had platelet-mediated microvascular dysfunctions (erythromelalgia) and 11 had major thrombotic events (arterial thrombosis: 8 cases, venous thromboembolism: 3 cases). The presence of cancer and the employment of chemotherapy agents increase the risk of cardiovascular events[13].

More than a half of ET patients were classified as intermediate risk (53.2%), followed by 34.6% in the high-risk group and 13.2% in the low- risk group. In the low- risk group, 6 patients were treated with low-dose aspirin 75 mg/day and 2 patients were put on watch-and-wait. In the intermediate risk group, 10 patients were prescribed low-dose aspirin 100 mg/day and 23 received low-dose of aspirin 100 mg/day + cytoreductive treatment. In the high-risk group, 12 cases were in treatment with low-dose aspirin 100 mg/day + cytoreductive treatment (hydroxyurea) and 9 cases received platelet-lowering agents (anagrelide).

ROS and TAC values were different based on the risk group and risk-adapted regimens employed. Mean values for ROS were: low-risk group = 2.18 mM/L, intermediate risk group = 2.43 mM/L and high-risk group = 2.63 mM/L. TAC values evolved oppositely:

Table 2. ROS and TAC evaluation before and after ET risk-adapted therapy

Risk group	*Before treatment*		*After treatment*		*p-value*
	ROS	CAT	ROS	CAT	
Low	2.18 ± 0.31	0.49 ± 0.21	1.86 ± 0.21	0.60 ± 0.26	p <0.05
Intermediate	2.43 ± 0.62	0.47 ± 0.16	1.92 ± 0.18	0.66 ± 0.28	p <0.05
High	2.63 ± 0.83	0.46 ± 0.10	1.96 ± 0.16	0.69 ± 0.29	p <0.05

low- risk group = 0.49 mM/L, intermediate risk group = 0.47 mM/L and high-risk group = 0.46 mM/L. After risk-adapted therapy was selected in ET cases, ROS levels decreased and TAC increased for all risk groups, as shown in **Table 2**. Data are presented as mean value ± standard deviation.

Discussion

In our study, we evaluated ROS and TAC values in patients diagnosed with ET vs. healthy controls. We found that ROS values are elevated and TAC is decreased in ET cases vs. controls and that risk-adapted therapy leads to a reduction in ROS numbers and also to an increase in antioxidant levels. The effect on oxidative stress, according to our data, was similar in ET patients treated with cytoreductive therapy (hydroxyurea) and platelet-lowering agents (anagrelide), with no statistically significant differences on oxidative stress levels between the two drugs.

Our results reinforce that oxidative stress is involved in chronic myeloproliferative neoplasms, as other authors have already suggested: ROS values were increased and TAC was decreased in patients with ET vs. healthy controls[7,12]. In ET patients that were prescribed cytoreductive treatment, according to a study published by Durmus et al, a significant decrease in oxidative stress parameters was registered after cytoreductive treatment. The same research suggested that oxidative stress markers were increased and TAC was decreased in ET cases vs. controls, as in our study[14]. Oxidative stress remains a hot topic in the field of cancer research, since its involvement has been proposed in many malignancies, as well as in other disorders. In the near future, the modulation of chemotherapy agents by natural products could emerge as a strategy to reduce toxicity and adverse effects of anticancer drugs, lower oxidative stress levels and increase antioxidant levels[15-18].

Conclusions

Our study revealed that ROS levels are increased and TAC is decreased in patients with ET vs. controls. Risk-adapted therapy in ET reduced ROS levels and increased TAC with high-risk patients registering the highest rise in TAC and the highest decrease in ROS values. No significant differences were seen regarding oxidative stress parameters in patients treated with cytoreductive treatment (hydroxyurea) and patients treated with platelet-lowering agents (anagrelide).

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article"

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study"

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