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*ORIGINAL ARTICLE*

# Oxidative stress in blood in cases of untreated refractory anaemia

N. Tzvetkov[1], P. Bochev[2], M. Alexandrova[2]
*[1]Clinic of Haematology, [2]Department of Biophysics, Medical University, Pleven, Bulgaria*

## Summary

***Purpose:*** *To examine some features of free radical processes in the blood of patients with untreated refractory anaemia (RA) and to interpret their pathogenetic role.*

***Patients and methods:*** *Products of the lipid peroxidation (malondialdehyde-MDA) in whole blood, some antioxidant systems – superoxide dismutase (SOD) in erythrocytes, catalase (CTS) activity and the concentration of sulfhydryl groups (SHG) in whole blood, as well as the spontaneous and stimulated chemiluminescent activity of polymorphonuclear leucocytes (PMNL), reflecting the production of oxygen free radicals, were studied in the blood of 21 patients suffering from untreated RA (study group) and in 45 healthy individuals (control group).*

***Results:*** *Increased content of MDA ($p < 0.001$), lowered level of CTS activity ($p < 0.001$) and concentration of SHG ($p < 0.05$), increased SOD activity ($p < 0.05$) and an increase in the spontaneous PMNL oxidative activity ($p < 0.05$) were found in the study group compared with the control group.*

***Conclusion:*** *Strong oxidative stress was recorded in the blood of patients with untreated RA. An assumption was made that the initial iron overload helps the initiation of free radical oxidative processes in blood, accompanied with spontaneous activation of PMNL. Erythrocyte membranes probably are the main target for oxidative attack.*

**Key words:** antioxidants, chemiluminescence, lipid peroxidation, myelodysplastic syndromes, oxygen free radicals, refractory anaemia

## Introduction

An important role for the development of myelodysplastic syndromes (MDS) plays the damaged apoptosis of immature bone marrow cells and the mature cells in the peripheral blood [1-4].

Amongst the probable reasons is the development of free-radicals processes in the blood cells [5]. Oxidative stress damages DNA in CD34+ cells in the bone marrow [6] and the function of bone marrow stroma [7], causes cytogenetic abnormalities [8], and damages apoptosis of haematopoietic bone marrow cells [6]. The positive results of the treatment of MDS with antioxidants such as amifostine are proofs for the crucial role of oxidative stress in the pathogenesis of MDS [5,9].

RA is one of the most widely encountered MDS. Ineffective erythropoiesis in cases of RA results in iron metabolism disorders, leading to chronic iron overload in the organism. Apart from this, there is a higher reabsorption of iron and inevitable blood transfusion requirements [10,11]. An increased content of serum iron and lower iron-binding capacity, increased saturation of transferrin, increased serum and erythrocyte ferritin, and presence of non-transferrin-bound redox-active iron in the serum are reported as early as RA is diagnosed [10,12]. After a continuous transfusion therapy even higher levels of the above mentioned parameters are reported [10,11]. The development of transfusional iron overload makes the prognosis worse and decreases patients' chance of living [13].

The chronic and progressing iron overload in

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*Author and address for correspondence:*

Nikolai Tzvetkov, MD
Medical University
Clinic of Haematology
University Hospital
First Base, 8 Georgi Kochev Street
5800 Pleven
Bulgaria
Fax: +359 64 806080
E-mail: tzvetkovn@yahoo.com

cases of RA creates conditions for the development of free-radical processes.

The aim of this study was to examine the existence of free radical processes in the blood of patients with newly diagnosed, untreated RA and to interpret their pathogenetic role in this condition.

## Patients and methods

*Study group*

Twenty-one untreated nonsmokers patients (13 men, 8, women) with RA, aged from 20 to 75 years (mean 45 ± 7.6) were examined. Their mean values of haemoglobin, serum iron and total iron-binding capacity were 71.0 ± 10.0 g/L, 35.5 ± 2.4 μkmol/L, and 43.3 ± 1.8 μkmol/L, respectively. The cytogenetic bone marrow analysis did not reveal any chromosome abnormalities. The patients selected did not have any other clinically or paraclinically expressed disease and did not take any drug during the study.

*Control group*

The control group consisted of 45 healthy asymptomatic nonsmokers (20 males, 25 females), aged from 18 to 58 years (mean 42 ± 5.3). Their mean haemoglobin was 134.0 ± 3.0 g/L, and the mean serum iron and total iron-binding capacity were 18.2 ± 4.6 μkmol/L and 61.5 ± 3.1 μkmol/L, respectively.

*Blood samples*

Five ml of whole fasting venous blood were taken for investigation. The haematological parameters were measured using Technicon H-1 Analyzer. Serum iron and the total iron-binding capacity were estimated by a colorimetric method with ferozyne (Roche Diagnostics, GmbH Roche Laboratory Systems, Mannheim, Germany).

*Lipid peroxidation products*

The level of lipid peroxidation in blood was estimated by the concentration of malondialdehyde (MDA) in whole blood, measured spectro-photometrically according to the Asakawa and Matsushita method [14]. The initially obtained data were normalized by the red blood cells count per milliliter of blood sample, since the major subject of peroxidation were the lipids of their membranes.

*Study of some antioxidant systems*

The antioxidant capacity of blood was estimated by determining the superoxide dismutase (SOD) activity in erythrocytes using the method of Maral et al. [15]. The results are presented per milliliter of blood sample. Since the number of erythrocytes in the blood samples are different, these data were additionally normalized according the erythrocyte number in the sample. The total catalase (CTS) activity of blood was measured by the method of Cohen et al. [16], and the total concentration of thiol groups (SHG) according to Ellman [17]. The results obtained are presented per milliliter of blood sample.

*Chemiluminescent studies of polymorphonuclear leukocytes (PMNL) oxidative activity*

Resting and stimulated oxidative activity of blood PMNL, which represents the generation of activated oxygen forms, were estimated by chemiluminescent method. The chemiluminescence from whole blood samples was measured by a 6-sample luminometer [18]. For the measurement of resting PMNL activity each sample contained 15 μkL of the investigated blood in 3 mL luminol solution (6.6 μkmol/L) in Krebs-Ringer phosphate buffer (pH 7.4). Luminol is used as luminescent enhancer. For stimulated PMNL activity 0.5 mL zymosan suspension (2 mg zymosan/mL) was added to each sample. Zymosan (purchased from Sigma), preliminary opsonised with pooled AB human serum, was used as stimulating agent. Each sample was tested simultaneously in triplicate. Since the concentrations of haemoglobin in erythrocytes and the number of PMNL in the blood samples are of great importance to the registered intensity of chemiluminescence, the initially acquired data in counts per min (cpm) were normalized according the PMNL number in the sample (cpm/$10^4$ PMNL) and corrected as to the optic absorbance of haemoglobin by the method of Ristola and Repo [19]. The chemiluminescent activity of PMNL of each sample was estimated by the area under the obtained kinetic chemiluminescent curve in relative units (RU) for a period of 30 min.

*Statistical analysis*

Statistical processing of the results obtained was carried out by the method of variation analysis. The data obtained are presented as mean value ± standard error. A p-value $<0.05$ was considered as statistically significant.

## Results

Lipid peroxidation in the study group displayed highly significant ($p < 0.001$) larger concentration of MDA compared to controls (Table 1). The antioxidant capacity in the study group (SOD activity in erythrocytes) was statistically increased ($p < 0.05$) and was combined with highly significant decreased CTS activity and concentration of blood sulfhydryl groups. The spontaneous oxidative activity of granulocytes was statistically increased ($p < 0.05$), but there was no change in their stimulated oxidative activity.

**Table 1.** Results of the examined parameters

| *Parameter* | *Control group* | *Study group* | *p-value* |
|---|---|---|---|
| MDA (nmol/L) | 4.76 ± 0.3 | 10.05 ± 1.2 | <0.001 |
| SOD (U/mL) | 3.20 ± 0.1 | 4.83 ± 0.1 | <0.05 |
| CTS (kU/mL) | 3.88 ± 0.1 | 1.51 ± 0.2 | <0.001 |
| SHG (μkmol/L) | 4.00 ± 0.2 | 2.96 ± 0.27 | <0.05 |
| Spontaneous chemiluminescence (RU) | 14.4 ± 2.7 | 28.8 ± 7.8 | <0.05 |
| Stimulated chemiluminescence (RU) | 148.9 ± 11.0 | 153.6 ± 25.3 | NS |

NS: nonsignificant
For abbreviations, see text

## Discussion

Growing evidence leaves little doubt that oxidative stress exists extra- and intracellularly in the bone marrow and the peripheral blood in cases of MDS.

It is observed that in different components of the blood cells the percentage of prooxidants (iron, tumor necrosis factor (TNF-α), homocysteine, oxygen free radicals) grows and the products of lipid peroxidation (lipid peroxides and MDA) are on the increase [5-8, 12,20].

In patients with RA Levina et al. [10] have found reduced SOD and CTS activity in erythrocytes, and decreased antioxidant activity of plasma. The authors explained these changes with the prooxidant effect of iron and recommended a simultaneous application of desferrioxamine and antioxidants. Fracchiolla et al. [8] also reported on abnormalities in the antioxidant potential of blood (glutathione, glutathione peroxidase, thiol groups, SOD, CTS) in various MDS. The glutathione level in bone marrow mononuclear cells was reported as lower [6]. Others, like Omata et al. [21] did not observe any changes in erythrocyte SOD activity of patients with MDS.

Many authors reported on decreased stimulated chemiluminescence activity of PMNL in MDS [22-25].

We assume that the oxidative stress in blood in cases of untreated RA is caused by the iron overload and the increased spontaneous oxidative activity of PMNL. The lipid peroxidation of the erythrocyte membranes (as the dominating cell component in blood) is a possible extra reason for the anaemic syndrome in RA. It is not clear what provokes the increase in the spontaneous oxidative activity of PMNL - products of lipid peroxidation, oxygen free radicals, redox-active iron, cytokines or other factors. There are proofs that the oxygen free radicals from the spontaneously activated PMNL make the release of iron from its protein complexes easier, especially in the presence of iron overload [26,27]. Khwaja et al. [28] found that TNF-α increases the stimulated oxidative activity of PMNL. It is possible that it also stimulates the spontaneous oxidative activity of PMNL.

The inevitable blood transfusion treatment, along with the ineffective erythropoiesis, aggravates the iron overload and hence the oxidative attack against blood cells. Jensen et al. [11,29] showed that continuous and systematic application of desferrioxamine reduces the requirements of transfusions in patients with MDS, while the number of platelets and granulocytes grows.

According to our study, the decrease of extra- and intracellular iron in the organism results in the reduction of oxidative stress on the blood cells and in the prolongation of their lifespan. An analogous effect can also be expected from the application of antioxidants.

## References


1. Shimazaki K, Ohshima K, Suzumiya J, Kawasaki C, Kikuchi M. Evaluation of apoptosis as a prognostic factor in myelodysplastic syndromes. Br J Haematol 2000; 110: 584-590.
2. Parker J, Mufti G. The role of apoptosis in the pathogenesis of the myelodysplastic syndromes. Int J Hematol 2001; 73: 416-428.
3. Claessens Y, Fontenay-Roupie M. Physiopathology of myelodysplastic syndromes. Pathol Biol 2002; 50: 261-267.
4. Emura I. High incidence of apoptosis in peripheral blood of myelodysplastic syndrome patients determined by Papanicolaou-stained preparations. Lab Hematol 2003; 9: 42-46.
5. Farquhar M, Bowen D. Oxidative stress and the myelodysplastic syndromes. Int J Hematol 2003;77: 342-350.
6. Peddie C, Wolf R, McLellan L, Collins A, Bowen D. Oxidative DNA damage in CD34+ myelodysplastic cells is associated with intracellular redox changes and elevated plasma tumor necrosis factor–α concentration. Br J Haematol 1997; 99: 625-631.

7. Tauro S, Hepburn M, Bowen D, Pippard M. Assessment of stromal function, and its potential contribution to deregulation of hematopoiesis in the myelodysplastic syndromes. Haematologica 2001; 86:1038-1045.
8. Fracchiolla N, Catena F, Novembrino C, Ippolito S, Maisonneuve P, Cortelezzi A. Possible association between oxygen metabolites and karyotypic abnormalities in myelodysplastic syndromes. Haematologica 2003; 88: 294-297.
9. Invernizzi R, Travaglino E, Gobbi P, Malabarba L, Ramajoli I, Ascari E. Clinical and biological effects of treatment with amifostine in myelodysplastic syndromes. Br J Haematol 2002; 118: 246-250.
10. Levina AA, Andreeva AP, Tsvetaeva NV, Tsibulskaya MM, Minaeva LM, Tokarev YuN. Antioxidant activity of serum and erythrocytes in patients with refractory anemia. Hematol Transfusiol 1991; 36: 11-14.
11. Jensen PD, Jensen IM, Ellegaard J. Desferrioxamine treatment reduces blood transfusion requirements in patients with myelodysplastic syndrome. Br J Haematol 1992; 80: 121-124.
12. Cortelezzi A, Cattaneo C, Cristiani S et al. Non-transferrin-bound iron in myelodysplastic syndromes as a marker of ineffective erythropoiesis. Hematol J 2000; 1: 153-158.
13. Gazzola M, Barosi G, Gobbi PG, Invernizzi R, Riccardi A, Ascari E. Natural history of idiopathic refractory sideroblastic anemia. Blood 1988; 71: 305-312.
14. Asakawa T, Matsushita S. Thiobarbituric acid test for detecting lipid peroxides. Lipids 1980; 14: 401-406.
15. Maral J, Puget K, Michelson A. Comparative studies of SOD, catalase and glutathione peroxidase in erythrocytes of different animals. Biochem Biophys Res Commun 1977; 77: 1525-1535.
16. Cohen G, Dembiec D, Marcus J. Measurement of catalase activity in tissue extracts. Ann Biochem 1970; 34; 30-38.
17. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82: 70-77.
18. Bochev P, Bechev B, Magrisso M. Six-sample multiplexing computerized analyser for integral and spectral luminescence measurements. Ann Chim Acta 1992; 256: 29-32.
19. Ristola M, Repo H. Whole blood luminol-enhanced chemiluminescence: Statistical analysis of the responses of different subjects. J Biolum Chemilum 1990; 5: 155-160.
20. Cortelezzi A, Francchiolla N, Bamonti-Catena F et al. Hyperhomocysteinemia in myelodysplastic syndromes: specific association with autoimmunity and cardiovascular disease. Leuk Lymphoma 2001; 41: 147-150.
21. 21.Omata F, Nakazawa H, Nakano M, Arimori S. Erythrocyte superoxide dismutase in various hematological diseases. Tocai J Exp Clin Med 1990; 15: 99-106.
22. Boogaerts MA, Nelissen V, Roelant C, Goossens W. Blood neutrophil function in primary myelodysplastic syndromes. Br J Haematol 1983; 55: 217-227.
23. Boogaerts MA, Meeus P, Verhoef G, Malbrain S, Van Hove L, Verwilghen RL. Differential effects of IL-3, GM-CSF and G-CSF on preleukemic granulocytes. Blood 1988; 72 (Suppl): 110-116.
24. Verhoef G, Boogaerts M. In vivo administration of granulocyte-macrophage colony stimulating factor enhances neutrophil function in patients with myelodysplastic syndromes. Br J Haemotol 1991; 79: 177-184.
25. Zwierzina H, Holzinger I, Gaggl S et al. Recombinant human interleukin-8 restores function in neutrophils from patients with myelodysplastic syndromes without stimulating myeloid progenitor cells. Scand J Immunol 1993; 37: 322-328.
26. Britigan BE, Edeker BL. Pseudomonas and neutrophil products modify transferrin and lactoferrin to create conditions that favor hydroxyl radical formation. J Clin Invest 1991; 88: 1092-1102.
27. Rosen GM, Pou S, Ramos LL, Cohen MS, Britigan BE. Free radicals and phagocytic cells. FASEB J 1995; 9: 200-209.
28. Khwaja A, Carver J, Linch D. Interactions of granulocyte-macrophage colony-stimulating factor (CSF), granulocyte CSF, and tumor necrosis factor alpha in the priming of neutrophil respiratory burst. Blood 1992; 79: 745-753.
29. Jensen PD, Heickendorff L, Pedersen B et al. The effect of iron chelation on haemopoiesis in MDS patients with transfusional iron overload. Br J Haematol 1996; 94: 288-299.