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OXIDATIVE STRESS IN PATIENTS WITH β-THALASSEMIA MAJOR

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Abstract: the aim of this work is to study the level of oxidative stress in blood of *β*-thalassemia major patients with transfusional iron overload and chelation therapy as a central pathological process.

β-thalassemia major results in an increase in the concentration of lipid peroxidation products in blood plasma of more than 100% and in the intensity of chemiluminescence – about 20% in comparison to healthy controls. The activity of the antioxidant enzyme superoxide dismutase in the blood of *β*-thalassemia major patients is decreased by more than 30% and the total antioxidant activity is diminished by about 70% compared to controls.

Experimental data confirm the progression of oxidative stress in patients with *β*-thalassemia major: activation of free radical processes and lipid peroxidation, decreased antioxidant capacity. Strong oxidative damage and essential alternations define these parameters as sensitive markers of oxidative stress in patients with *β*-thalassemia major. The combination of effective iron-chelatory agents with natural or synthetic antioxidants can be extremely helpful in clinical practice in the regulation of the antioxidant status of patients with *β*-thalassemia major.

Key words: *β*-thalassemia major, oxidative stress, lipid peroxidation, antioxidant activity, blood.

Introduction

Thalassemic syndromes are a group of hereditary and severe disorders, resulting from the homozygous state of one of the thalassemia or hemoglobin Lepore genes in infancy or childhood [1, 2]. It is accompanied with metabolic irregulation, iron overload, chronic hypoxia and cell damage [2]. All the physiological changes result in ineffective erythropoiesis, haemolysis and anaemia [3].

Oxidative stress is an important mechanism in the progression of *β*-thalassemia major and in many other diseases such as cardiovascular failure, cancer, renal and neurological diseases, infections, etc. [1, 4, 5]. This process is characterized by metabolic hyper-production of reactive oxygen species (ROS) and induced lipid peroxidation (LPO). Oxidative stress exceeds the capacity of the antioxidant defences (content of antioxidants and activity of antioxidant enzymes). It activates diverse damaging processes in cells, including oxidation of intracellular and surface components of the red blood cells in *β*-thalassemia major patients [6, 7, 8].

Typical treatment of β-thalassemia major consists of multiple blood transfusions, a complication of which is iron overload [9]. Early introduction of chelatory agents control and combat iron overload, inhibit ROS-generation and regulate LPO-processes, leading to improved life expectancy [10].

The aim of this work is to study the level of oxidative stress as a central pathological process in the blood of *β*-thalassemia major patients with transfusional iron overload and chelation therapy by registering clinical data, concentration of LPO-products and chemiluminescence, total antioxidant activity (TAOA) and superoxide dismutase (SOD) activity.

Materials and Methods

Patients: Blood samples were obtained from 22 *β*-thalassemia major patients (10 female and 12 male subjects, age 5–21). They were recruited from the Traque University Hospital, Stara Zagora, Bulgaria. The patients had suffered from *β*-thalassemia major for various periods of time, but all had been treated conventionally. They needed no blood transfusion for at least 1 month before donating the blood samples for these studies and were asked to take no medications except their daily Desferral supplementation. The examined patients did not suffer from any concomitant disease. Blood transfusions supported physiological haemoglobin concentrations and Desferral therapy corrected the ferritin content. Blood plasma obtained from 17 subjects (8 female and 9 male, age 5–13) was used as healthy controls. None of the patients or control subjects enrolled in this study received antioxidant supplementations that could affect the results.

This study was approved by the Ethics Committee. The investigation conformed to the principles outlined in the Helsinki Declaration (Br Med J 1964; ii:

177). The children's parents and the adult patients gave informed consent to blood sampling.

Blood samples required for this study were obtained by means of sterilized plastic syringes (total volume 20 ml). Blood was collected in heparinized sterilized tubes and centrifuged $15min_x2000g$ at 4 ^{o}C to obtain plasma.

Lipid peroxidation products: Thiobarbituric reactive substances (TBARS) were measured spectrophotometrically, according to the method of Asakawa T and Matsushita S, 1980 [11]. Samples contained 0.1 ml blood plasma, 10^{-2} M Fe_2SO_4, 10^{-2} M ionol (4-methyl-2,6-ditertbutyl-phenol), 0.2 M glycine-hydrochloric buffer, pH 3.6/25 ^{0}C and 0.5% TBA-reagent (0.5 g TBA and 0.3 g SDS). The reactive mixture was heated for 20min/100^{o}C. 1 ml ice-cold CH_3COOH was added after cooling the samples. The chromogen was extracted within 2 ml chloroform, after centrifugation. The malone dialdehyde–TBA complex was measured spectrophotometrically at 532 nm.

Activated Chemiluminescence: Chemiluminescence (CL) of blood plasma was measured for three minutes with a 0.2 M phosphate buffer, pH 7.4/25 ^{o}C, in an $O_2^{\cdot-}$-generating (NADH (10^{-4}M) – FMS (10^{-6} M)) system. Lucigenin (10^{-4} M) enhanced chemilumenescent activity registered the prooxidant activity at 470 nm (luminometer LKB 1251, Sweden).

Antioxidant Activity

Superoxide dismutase activity (SOD) was determined according to the modified method of Geller BL and Winge DR, 1984 [12]. After the separation of blood plasma, the packed erythrocytes were washed twice with 0.9% NaCl solution and haemolysed with ice-cold water. The total volume was 2 ml: 0.1 ml sample; 0.2 M phosphate buffer, pH 7.8/37^{o}C; 50 mM Na_2CO_3; 0.1 mM EDTA; 0.25 mM NBT; 10^{-4} M NADH; 10^{-6} M FMS. Measurements were performed spectrophotometrically at 560 nm.

Total antioxidant activity (TAOA) was determined according to the method of Koracevic D *et al*., 2001 [13]. A standard solution of uric acid was applied as a reference of the antioxidant activity. Blood plasma samples were measured spectrophotometrically at 532 nm.

The protein content in blood plasma was measured according to the method of Lowry OH *et al*., 1951 [14].

All measurements were performed in triplicate within 4–6 hours after blood sampling. The chemicals used for the analyses were purchased from Aldrich (USA) and Merck (Germany).

Statistical Analysis: Results were presented as mean $\pm$ standard deviation of the mean (STDEV). Statistically significant differences ($p \leq 0.05$) were determined with the Student's *T* test. Correlation coefficients were calculated according to the Brave-Pearson function.

Results

β-thalassemia major was accompanied by the generation of LPO-products in the blood plasma of conventionally treated patients. The concentration of TBARS was increased by more than 100%, compared to healthy controls (Figure 1A).

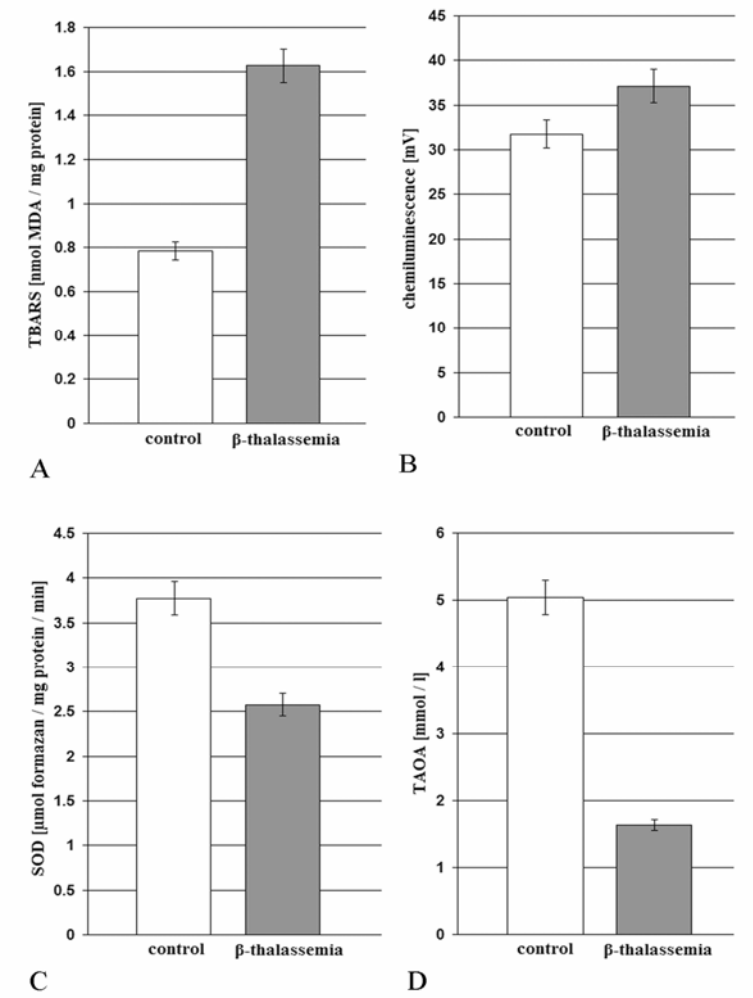

Figure 1 – TBA-reactive substances (A), intensity of chemiluminescence (B), total antioxidant activity (D) in blood plasma and superoxide dismutase activity (C) in blood of patients with β-thalassemia major ($p \leq 0.05$)

Слика 1 – ТВА-реактивни супстанци (А), интензитет на хемилуминисценција (В), тоталана антиоксидантна активност (D) во крвната плазма и супероксид дизмутаза (С), кај пациенти со β-таласемија мајор

The intensity of chemiluminescence in the blood plasma of *β*-thalassemia major patients was about 20% higher in comparison to the controls (Figure 1B).

The activity of the antioxidant enzyme SOD was decreased by more than 30% in the blood of β-thalassemia major patients (Figure 1C).

B-thalassemia major caused low antioxidant defence in these patients. TAOA was diminished by about 70% compared to healthy subjects (Figure 1D).

Clinical data confirmed typical symptoms in all the examined *β*-thalassemia major patients: a total decrease of the haemoglobin and hematocrit content, as well as the number of red blood cells, mean cell volume and mean corpuscular haemoglobin value (Table 1).

Table 1 – Табела 1

Clinical parameters in β-thalassemia major patients, before haemotransfusion
Клинички параметри кај пациенти со β-таласемија мајор, пред хемотрансфузија

Healthy control	Parameter	*β*-thalassemia major patients					
		Average	STDEV	Average STDEV	Min.	Max.	Ve%
12–14	Hb [g/dl]	6.28	1.54	0.76	4.60	8.96	2.45
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0.36–0.47	HCT [l/l]	0.17	0.04	0.02	0.12	0.24	24.47
34–38	MCH [pg]	26.71	2.68	1.32	23.00	31.40	10.40
82–92	MCV [fl]	71.99	4.58	2.28	64.10	82.00	6.36
1.20–13.70	Reticulocytes [10^9/l]	23.46	4.86	1.06	14.20	31.20	19.90
3.50–10.50	WBC [10^9/l]	7.10	1.29	0.28	4.60	10.20	18.20
10.70–23.30	Serum iron [μmol/l]	24.86	9.15	1.48	12.30	43.20	36.80

Discussion

The anaemia in patients with β-thalassemia major is caused by a combination of haemolysis in peripheral blood circulation and ineffective erythropoiesis. Studies of haemolysis have indicated that oxidant injury to circulating erythrocytes is of critical importance, with evidence of oxidative stress in red blood cell proteins [2, 7]. Oxidant damage to thalassemic erythroid precursors can cause

their accelerated apoptosis and ineffective erythropoiesis [15]. It appears that oxidative stress is a basic mechanism in β-thalassemia major pathological alternations.

It has already been established that oxidative stress is increased in patients with iron overload (Table 1). This is a result of the mounted concentration of highly reactive Fe^{2+} ions which catalyze the Fenton's and Haber-Weiss reactions and the generation of ROS. They initiate the process of autocatalytic free radical lipid peroxidation generating a large variety of potential genotoxic breakdown products, including alkoxyl radicals, peroxyl radicals and aldehydes, such as malonedialdehyde. The level of TBARS in the investigated *β*-thalassemia major patients was raised by more than 100% (Figure 1A) [16, 17, 18]. This result illustrates that the concentration of LPO-products is a proper marker, suitable for express estimations of oxidative stress in clinical manifestations of the disease [16].

The activation of LPO-processes is accompanied by generation of oxygen radicals and radical intermediates. Chemiluminescence is an express quantitative method for the registration of the extremely fast radicals' recombination and primal content as well as the kinetics of these processes. Lucigenin enhanced chemiluminescence of blood plasma in the (FMS-NADH) system demonstrates high pro-oxidant activity in patients with *β*-thalassemia major (Figure 1B).

Antioxidant capacity is a result of the overall effect of water-soluble antioxidants, lipid-soluble antioxidants and antioxidant enzymes such as superoxide dismutase. Oxidative stress is caused by prolonged imbalance and antioxidant depletion as well as hyper-production of ROS [19]. SOD-activity is an indirect method for registration of the content of primary ROS ($O_2^{\cdot}$ radicals). SOD activity in patients with *β*-thalassemia major is decreased by more than 30% (Figure 1C), resulting in pronounced inhibition of the blood antioxidant capacity. This is confirmed by the negative correlation between the activity of SOD and the concentration of TBARS ($r = -0.698$). Our data are also supported by other investigations [20]. Although the results achieved by different authors are controversial, a possible explanation is the inhibition of SOD and cytoplasmic enzymes by free Hem in *β*-thalassemia major [1, 6, 21]. The accelerated ROS-production also causes the expression of several specific metal-binding proteins (transferrin, cerruloplasmin, ferritin, lactoferitin, etc.) and subsequent progression of oxidative stress [22].

Since antioxidants seem to act co-operatively *in vivo*, the evaluation of TAOA in blood plasma could provide a more comprehensive assessment than the evaluation of individual antioxidants. TAOA is a parameter, summarizing the overall content and activity of the water-soluble antioxidants. The depletion of TAOA induced by oxidative stress in *β*-thalassemia major patients is probably eliminated by the release of stock organ antioxidants and the induction or

activation of antioxidant enzymes [23]. B-thalassemia major suppresses antioxidant defence by about 70% (Figure 1D), causing oxidative stress and exhaustion of metabolic antioxidants.

Free radical processes and oxidative stress induced by Fe^{2+} ions in *β*-thalassemia major patients are inhibited by complex-forming agents such as Desferral, Apo-transferin, Apo-lactoferin, etc. [24]. Iron complexes so formed exhibit a pro-oxidant or antioxidant effect depending on the reactive conditions. Three basic active mechanisms and complex forming agents are distinguished:

1) Agents diminishing Fe^{2+} active concentrations (e.g. phenatropin)

2) Water-soluble complex forming agents (e.g. Fe^{2+}-ADP)

3) Agents changing Fe^{2+}/Fe^{3+} oxidative-reductive capacity (e.g. Fe^{2+} and Desferral) [22].

Clinical data confirm that the constant decrease of the haemoglobin level is accompanied by a decrease in the erythrocytes number and by diminished values of their specific indexes (MCV, MCH, MCHC, HCT, etc.). The content of serum iron (24.9 μmol/l) and ferritin (2 300–4 000 pg/ml) was increased above that of the controls in all the patients examined. This increase was probably determined by inefficient Desferral therapy. Higher ferritin content was directly linked to the accumulation of reactive iron in the tissues of these patients. Iron overload starts another pathological mechanism leading to oxidative damage of erythrocytes membranes, the so-called "second disease" [3].

Our experimental data confirm the progression of oxidative stress in conventionally treated *β*-thalassemia major patients: an increased level of LPO-products and an increased concentration of free radical intermediates and chemilumenescent intensity accompanied by the simultaneous inhibition of the antioxidant capacity (decreased TAOA and SOD activity). Strong oxidative damage and sharp alternations define these parameters as sensitive markers of oxidative stress in patients with *β*-thalassemia major. They can easily follow the dynamics and complications of the disease. The combination of effective iron-chelatory agents with natural or synthetic antioxidants can be very helpful in clinical practice and the regulation of the antioxidant status of patients with *β*-thalassemia major.

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Резиме

ОКСИДАТИВЕН СТРЕС КАЈ ПАЦИЕНТИ СО *β*-ТАЛАСЕМИЈА МАЈОР

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Целта на овој труд е да се проучи нивото на оксидативен стрес во крвта кај пациенти со *β*-таласемија мајор кои истовремено се оптоварени со железо (како резултат на примање на трансфузии) и поради тоа се на терапија со хелати.

β-таласемија мајор резултира со зголемување на концентрацијата на продукти на липидна пероксидација во крвната плазма за повеќе од 100% и во зголемување на интензитетот на хемилуминисценција за околку 20% споредено со здравите индивидуи. Активноста на супероксид дизмутазата (антиоксидантен ензим) во крвта на пациенти со *β*- таласемија мајор е намалена за повеќе од 30%, а тоталната антиоксидативна активност е намалена за 70% споредено со контролната група.

Експерименталните податоци ја потврдуваат прогресијата на оксидативниот стрес кај пациенти со *β*-таласемија мајор – активација на процеси кои вклучуваат слободни радикали и липидна пероксидација и намален антиоксидататиевен капацитет. Силното оксидативно оштетување, како и суштинските алтерации во вредноста, ги дефинираат овие параметри како осетливи маркери на оксидативниот стрес кај пациенти со *β*-таласемија мајор. Комбинацијата на ефикасна хелатна терапија со природни или синтетски антиоксиданси може да е од големо

значење во клиничката пракса со цел регулацијата на антиоксидантниот статус кај пациенти со β-таласемија мајор.

Клучни зборови: β-таласемија мајор, оксидативен стрес, липидна пероксидација, антиоксидативна активност, крв.

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Table 1 – Табела 1

Clinical parameters in β-thalassemia major patients, before haemotransfusion
Клинички параметри кај пациенти со β-таласемија мајор, пред хемотрансфузија

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10.70–23.30	Serum iron [μmol/l]	24.86	9.15	1.48	12.30	43.20	36.80

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