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Oxidative stress modulates the organization of erythrocyte membrane cytoskeleton

Wpływ stresu oksydacyjnego na białka błon erytrocytów

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Apart from their main role in transporting oxygen and carbon dioxide, erythrocytes play also an important role in organism antioxidative defence. Direct exposure to reactive oxygen species (ROS) results in shortening of their half-life, even by 50%. The presence of glucose, being the substrate in pentose phosphate pathway (PPP) cycle, is one of the factors that can have influence on the level of oxidative stress. The activity of PPP increases during oxidative stress. Glucose guarantees normal PPP functioning with the production of reductive equivalents in the amounts necessary to reproduction of glutathione – nonenzymatic free radical scavenger. In available literature there are no reports regarding the changes in protein contents of erythrocyte cytoskeleton exposed to t-butyl hydroperoxide in relation to glucose presence in incubation medium.

Material/methods: Erythrocytes taken from 10 healthy subjects were used to assess the influence of generated free radicals on erythrocyte proteins and chosen parameters of oxidative stress. Erythrocytes were incubated in the solutions containing deferent concentrations of t-butyl hydroperoxide and glucose. Electrophoresis was performed on polyacrylamide gel in denaturating conditions. The contents of tryptophan in membranes was evaluated spectrofluorometrically.

Results/conclusions: *In vitro* conditions oxidative stress leads to protein damage in erythrocyte cytoskeleton, both in proteins inside the cell as well as having contact with extracellular environment. In consequence, the amount of low-molecular proteins – mainly globin, which bind to cytoskeleton, increases. This process takes place independently of glucose presence in incubation medium. One of the element of protein cytoskeleton, tryptophan, also undergoes degradation. The decrease of its contents is higher during erythrocyte exposure to t-BOOH in environment containing glucose, what can suggest prooxidative influence of glucose in conditions *in vitro*.

Key words: **oxidative stress • erythrocytes • cytoskeleton proteins**

Streszczenie

Wstęp: Krwinki czerwone, oprócz swej podstawowej roli związanej z przenoszeniem tlenu i dwutlenku węgla są komórkami odgrywającymi doniosłą rolę w obronie antyoksydacyjnej organizmów żywych. Bezpośrednie narażenie na reaktywne formy tlenu skutkuje skróceniem, nawet o 50% czasu życia krwinek. Jednym z czynników mających wpływ na poziom stresu oksydacyjnego jest

obecność glukozy, będącej m.in. substratem w szlaku pentozofosforanowym (PPP), którego aktywność wzrasta w warunkach wzmożonego stresu oksydacyjnego. Gwarantuje ona prawidłowe działanie cyklu PPP, czego konsekwencją jest wytwarzanie równoważników redukcyjnych w ilościach niezbędnych do zapewnienia odtwarzania glutationu – nieenzymatycznego zmiatacza wolnych rodników. W dostępnej literaturze nie ma doniesień różnicujących zmiany w składzie białkowym cytoszkieletu erytrocytów traktowanych nadtlenkiem t-butylu w zależności od obecności glukozy w medium inkubacyjnym.

Materiał/metody: Do badań wpływu generowanych wolnych rodników na białka erytrocytów i wybrane parametry stresu oksydacyjnego stosowano krwinki czerwone pobrane od 10 zdrowych osób. Erytrocyty inkubowano w roztworach zawierających w różnych stężeniach nadtlenek t-butylu oraz glukozę. Rozdziału elektroforetycznego białek błon dokonywano na żelu poliakrylamidowym w warunkach denaturujących. Zawartość tryptofanu w błonach oznaczano spektrofluorymetrycznie.

Wyniki/wnioski: W warunkach *in vitro* stres oksydacyjny powoduje uszkodzenia białek cytoszkieletu erytrocytów obecnych tylko wewnątrz komórki, jak i mających kontakt ze środowiskiem pozakomórkowym. W konsekwencji zwiększa się ilość białek niskocząsteczkowych, głównie globin, które wiążą się do cytoszkieletu. Proces ten występuje niezależnie od obecności glukozy w medium inkubacyjnym. Degradacji ulega także tryptofan będący składnikiem białek cytoszkieletu. Obniżenie jego zawartości jest większe podczas traktowania krwinek t-BOOH w środowisku zawierającym glukozę, co może świadczyć o jej prooksydacyjnym działaniu w warunkach *in vitro*.

Słowa kluczowe: **stres oksydacyjny • krwinki czerwone • białka cytoszkieletu**

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Abbreviations: **1,3-BPG** – 1,3-bisphosphoglyceric acid; **DTT** – dithiothreitol; **G-3-PD** – glyceraldehyde-3-phosphate dehydrogenase; **G-6-P** – glucose-6-phosphate; **G-6-PD** – glucose-6-phosphate dehydrogenase; **PBS** – buffered solution of sodium chloride; **PPP** – pentose phosphate pathway; **ROS** – reactive oxygen species; **SDS** – sodium lauryl sulfate; **t-BOOH** – tert-butyl hydroperoxide.

Introduction

Oue earlier studies on erythrocytes from patients with chronic kidney disease undergoing hemodialysis treatment showed that the presence of glucose in dialysate led to changes in erythrocyte metabolism. The presence of glucose has influence on glutathione antioxidative system. It leads to activation of pentose phosphate pathway and production of reductive equivalents necessary to reproduction of reduced glutathione [8, 9], it also changes the energetic status of the cell [7]. It increases the intensity of oxidative stress, but it also decreases the ability of erythrocytes to hemolysis [23]. We confirmed theses changes with experiments *in vitro* and showed that during erythrocyte exposure to t-BOOH (tert-butyl hydroperoxide) in medium not containing glucose the concentration of reduced glutathione and the activity of superoxide dismutase decreased [8].

Chronic hyperglicemia induces mechanisms causing excessive production of free radicals. Glucose may undergo autooxidation (glycoxidation) and it intesifies the process of non-enzymatic glication of proteins. Numerous toxic oxygen derivatives are produced in this process.

In available literature there are no reports regarding the changes in protein contents of erythrocyte cytoskeleton exposed to t-butyl hydroperoxide in relation to glucose presence in incubation medium.

Erythrocyte membrane

Erythrocytes are highly specialized cells transporting oxygen to tissues and removing carbon dioxide. Life span of these cells, devoid of mitochondria and nuclei, as well as ribosomal mechanism of protein synthesis, is approximately 120 days. The apparent simplicity of their structure is mistaking. Erythrocytes are adapted to their functions. They contain viscous 'liquid crystal' – hemoglobin – surrounded by protein skeleton connected with lipid bilayer. The complex structure of the membrane contains lipid bilayer, attached proteins and peripheral proteins forming membrane skeleton. Direct interactions between some proteins of the skeleton and lipid bilayer are additional stabilization [1,52].

Membrane proteins can perform a wide diversity of functions, such as the role in transporting, adhesion, signaling. They also can exhibit enzymatic activity. Band 3 is the main membrane transporting protein. It makes up to 25% of the cell membrane surface. This anion exchanger protein also binds membrane skeleton to erythrocyte membrane. It is also the main place where hemichromes and hemoglobin bind to erythrocyte membrane. N-terminal domain consists of 403 amino acids and is anchored in cytoplasm due to connection with ankyrin and proteins 4.1 and 4.2. Terminal part of this domain, containing 23 amino acids, binds hemoglobin and glycolytic enzymes [56].

Membrane skeleton proteins make up to 60% of erythrocyte membrane internal surface. Spectrin, actin, band 4.1 protein, ankyrin and adducin are the main components [5,6,42]. Spectrin, the most prominent component of erythrocyte membrane skeleton, has two isoforms (alpha and beta) which form a loosely wound helix. Two alpha--beta helixes are linked end to end to form a single tetramer which has binding sites for actin microfilaments forming network on cytoplasmatic surface of the membrane. Ankyrin molecule is composed of three functional domains, two of which contain binding places for band 3 protein, spectrin, tubulin and intermediate filament proteins. The third functional domain regulates ankyrin binding to spectrin and band 3 protein [20]. Band 5 protein, actin, binds to spectrin and 4.1 protein [45]. 4.1 protein is a globular protein bound to spectrin close to the place of actin binding. These three proteins stabilize horizontal structure of erythrocyte cytoskeleton. 4.2 protein, bound to band 3 protein, shows similarity to transglutaminases, but without their activity. The role of 4.2 protein is not unequivocally determined. Band 7 protein is not homogenous, during two-dimensional electrophoresis it divides into at least 10 fractions, of which stomatin (7.2) is the most important. Its deficiency causes stomacytosis, the disease associated with excessive permeability of cellular membrane [19]. Band 8 has not been well known, however, it was found that its increased amount was associated with higher concentration of globin bound to membrane in patients with anaemia [3]. Glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) (band 6) is one of the three erythrocyte membrane proteins with enzymatic activity, one of the glycolysis enzymes, which catalyses the transformation of 3-phosphoglyceric aldehyde to 1,3-bisphosphoglyceric acid (1,3-BPG). G-3-PD is bound to cytoplasmatic domain of band 3 protein [51].

Band 9 protein, globin, is a small-molecule protein generated as a result of hemoglobin degradation. It is bound to erythrocyte cytoskeleton, frequently to spectrin. The amount of the protein increases during echinocyte transformation of erythrocytes [47], in stress, and in hereditary spherocytosis [40].

Oxidative stress. Its generation and indices

During the last decades studies have shown that reactive oxygen species (ROS) can be substrates, products and factors modulating many biochemical processes in human body. In physiological conditions the amount of free radicals generated in such processes as oxidation in mitochondrial respiratory chain, oxyhemoglobin autooxidation and oxidases activity, is balanced with the action of antioxidative systems. Reactive oxygen species include highly reactive hydroxyl radical, superoxide anion radical, superoxide radicals and compounds which are not free radicals: hydrogen peroxide and hypochlorous acid. Blood elements such as proteins and lipids, are the most exposed elements to free radicals. Oxidative stress leads to increased amounts of oxygenated and carbonyl derivatives of proteins [18,32].

Intensity of oxidative stress can be measured as the concentration of the products of the reaction of reactive oxygen forms with biomolecules: proteins, lipids, nucleic acids [16,17,25,34,54]. The consequence of free radical degradation of unsaturated fatty acids is the increased concentration of malonic aldehyde, arachidonic acid – isoprostanoids, nucleic acids – 8-Oxo-7,8 –dihydroguanine, and proteins – especially of cell membrane – tryptophan. Studies *in vitro* on hamster fibroblasts showed that oxidative stress induced by t-BOOH led to decreased concentrations of tryptophan in cell membranes [41]. Pernitrite in erythrocyte membrane also causes decrease of tryptophan concentration, which is inhibited when melatonin is added. Tryptophan deficiency was accompanied by decreased amount of spectrin in cell membrane [21].

Some aspects of erythrocyte metabolism

In oxidative stress erythrocyte uses 1.7-fold more glucose than in basal state [60]. Erythrocytes are circulating scavengers. Antioxidative functions of mature erythrocyte are directly related to intracellular glucose metabolism. Increased glucose metabolism is a result of increased erythrocyte metabolism. After penetration to erythrocyte, glucose undergoes phosphorylation to glucose-6-phosphate (G-6-P), which is metabolised in two competitive pathways: glycolysis or hexose monophosphate pathway/pentose cycle (PPP). Glycolysis is the main source of energy for erythrocytes. The second pathway, pentose cycle, is the only source of NADPH in erythrocytes. In physiological conditions, only a few percent of glucose-6-phosphate is consumed in PPP, whereas during oxidative stress, when NADPH is needed, the activity of this cycle increases even 20-fold [48]. The activity of PPP is regulated by the first enzyme in the cycle, glucose-6-phosphate dehydrogenase (G-6-PD), which is controlled, in turn, by the ratio of $NADP^+$/NADPH. High concentrations of $NADP^+$ facilitate the production of dimers and activation of G-6-PD, what places glucose in hexose monophosphate cycle. The consequence of G-6-PD deficiency in erythrocyte is decreased concentration of reduced glutathione and increased concentration of its oxidized form and increased amount of the products of lipid oxidization.

Because of cytoplasmic antioxidant systems, erythrocytes are assumed to be free radical scavengers. However, there is also a source of reactive oxygen species in erythrocytes. Hemoglobin concentration in erythrocyte is approximately 5 mmol/L, and the concentration of heme iron is approximately 20 mM. Cytoplasm of RBC is rich in oxygen, which, in the presence of ions of transient metals, mainly iron and copper, transforms to reactive forms, mainly free radicals. If the complex of oxygen and iron ion (Fe^{+2}) of deoxyhemoglobin is produced, it is possible for the electron from the outer shell of iron ion (II) to jump to oxygen

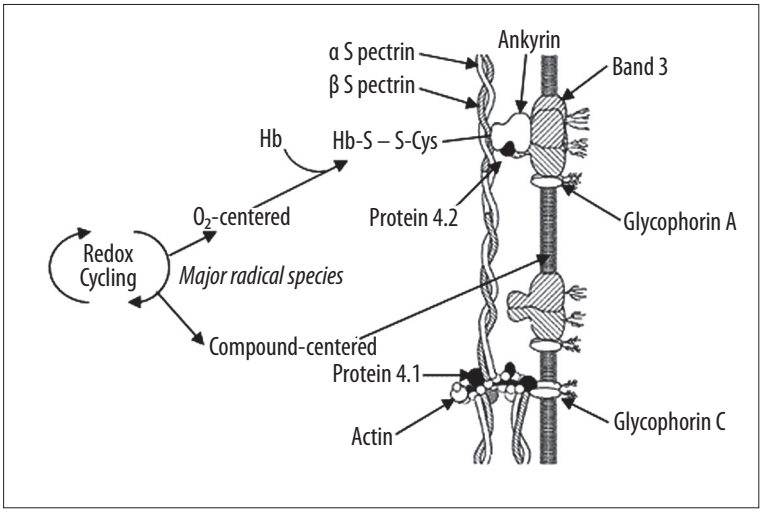

Fig. 1. The mechanism of globin binding to cytoskeleton proteins during exposure to reactive oxygen species [according to 33, modified]

atom. This produces methemoglobin and superoxide anion radical, which attacks thiol groups of hemoglobin and cytoskeleton proteins. As a result protein aggregates develop with hemoglobin residues and proteins with disulphide bond (fig. 1) [33].

The aim of our study was to assess if glucose presence in incubation medium had influence on structural elements of cytoskeleton of erythrocytes exposed to t-butyl hydroperoxide as a source of reactive oxygen species *in vitro*.

Material and methods

Erythrocytes from 10 healthy subjects were taken to assess the influence of generated free radicals on proteins and chosen parameters of oxidative stress.

10 mL of blood was drawn using heparin as anticoagulant (50 IU/mL). Blood was centrifuged (1850 g, 4°C, 10 min) and cells were separated from plasma. After leukocyte coat removal, cells were washed 3 times with buffered solution of sodium chloride (PBS) containing: NaCl 150 mmol/L, phosphate buffer 5 mmol/L; pH 7.4 in 4°C. The suspension of washed erythrocytes (hematocrit 5–7%) was in the solution with ingredients: glucose 0 or 5 mM, NaCl 150 mM, phosphate buffer 5 mM (pH 7.4 in 4°C). Tert-butyl hydroperoxide activity started after adding such amount of t-BOOH to achieve its concentrations of 0.1 and 1 mmol/L. Simultaneously, RBC were incubated in the solution with the same content, but without t-BOOH.

The suspension of erythrocytes was incubated for 1 hour in 37°C, periodically shaken. Then reaction mixture was cooled after placing it in cold (4°C) tubes, centrifuged at 1850 g, in 4°C for 10 min. Cells were washed 3 times with PBS and then freezed.

Membranes of erythrocytes were prepared according to Dodge [22]. Cells underwent lysis after they were washed in cold solutions of sodium phosphate in concentrations: 20, 10 and 5 mmol/L. The suspension of membranes was stored in –80°C. Protein separation was performed on polyacrylamide gel in denaturing conditions according to Laemmli [36]. Membranes of erythrocytes were suspended in the solution containing TRIS-HCl buffer 0.25 M, pH 6.8, SDS (sodium lauryl sulfate) 10% and DTT (dithiothreitol) 0.5 M and incubated in temp. 94°C for 5 minutes. Condensing gel 4% and analytic gel 10% were used. Protein separation was done in room temperature, 60V for condensing gel and 120 V for analytic gel. Gels were stained with Coomasie Briliant Blue 0.1% solution in acetic acid 10% and methanol 50%.

Spectrofluorometric determination of tryptophan amounts was performed on spectrofluorometer LS 50 B (Perkin-Elmer). The suspension of erythrocyte membranes used for analysis had protein concentration of 50 µg/mL. After excitation of protein solution at wavelength within the range 279–298 nm, fluorescency emision was measured at wavelength 320–350 nm.

Kits used in the study were bought in Sigma (USA). To obtain solutions, water was produced as a result of reverse osmosis on Milipore apparatus. Protein electrophoresis was performed on Mini Protean II (Bio-Rad). Quantitative analysis of densitograms was done using GelScan programme (Kucharczyk TE, Warsaw).

Statistical analysis

All results are presented as mean ± standard deviation (SD). As the distribution of obtained results was not normal (Shapiro-Wilk's test), following non-parametric tests were used: Wilcoxon's pair test for differences in parameters and Spearman's test to examine correlations between parameters.

Statistical analysis was performed using software Statistica (StatSoft Kraków).

Results

Obtained results and statistical relationships are presented in table 1, and figures 2 and 3. All values in tables are presented as arithmetic mean ± standard deviation.

Changes of erythrocyte membrane elements *in vitro*

No statistically significant differences were found in protein and tryptophan concentrations between erythrocytes

Table 1. The amounts of tryptophan and proteins of erythrocyte membrane [mg/100 mg of protein] exposed to t-BOOH in concentrations 0.1 and 1.0 mmol/L in solutions with and without glucose. Values of p parameter (Wilcoxon's test) in comparison between the groups of protein and tryptophan concentrations in conditions *in vitro*

Conditions / Parameter	Without glucose					With glucose					With glucose *vs* without glucose	
	t-BOOH concentration [mmol/L]			p value		t-BOOH concentration [mmol/L]			p value		t-BOOH concentration [mmol/L]	
	0.00	0.1	1.00	1.0 *vs* 0	1.0 *vs* 0.1	0.00	0.1	1.00	1.0 *vs* 0	1.0 *vs* 0.1	0.1	1.0
Spectrin	30.81 ±2.28	31.1 ±2.73	24.9 ±3.4	0.009	0.005	31.1 ±2.43	31.6 ±3.52	26.06 ±2.32	0.005	0.007	ns	ns
Band 3	36.89 ±2.03	35.49 ±1.90	40.1 ±2.2	0.01	0.005	36.1 ±1.91	35.2 ±2.57	39.14 ±1.73	0.009	0.009	ns	ns
Band 4.1	8.78 ±1.84	9.15 ±1.06	7.27 ±1.55	ns	0.03	8.54 ±1.09	9.25 ±1.42	7.99 ±1.57	ns	ns	ns	ns
Band 4.2	5.15 ±0.64	5.14 ±0.59	5.06 ±1.76	0.02	ns	5.27 ±0.74	5.24 ±0.62	4.67 ±0.84	ns	ns	ns	ns
Band 5	5.67 ±0.55	5.61 ±0.48	5.52 ±0.79	ns	ns	6.08 ±0.38	5.67 ±0.59	5.45 ±0.76	ns	ns	ns	ns
Band 6	5.17 ±0.95	5.58 ±0.77	5.67 ±0.96	ns	ns	5.87 ±0.64	5.61 ±0.96	5.78 ±0.84	ns	ns	ns	ns
Band 7	3.26 ±0.71	3.41 ±0.74	3.56 ±0.98	ns	ns	3.19 ±0.69	3.45 ±0.74	3.12 ±0.94	ns	ns	ns	ns
Band 8	2.03 ±0.57	2.15 ±0.62	2.99 ±0.50	0.005	0.005	1.67 ±0.51	1.87 ±0.68	2.67 ±0.36	0.005	0.005	0,020	ns
Globin	2.22 ±0.66	2.37 ±0.52	4.78 ±1.61	0.005	0.005	2.2 ±0.74	2.13 ±0.81	5.09 ±0.82	0.005	0.005	ns	ns
Band 4.1/spectrin	0.28 ±0.06	0.29 ±0.04	0.3 ±0.09	ns	ns	0.277 ±0.04	0.29 ±0.06	0.31 ±0.08	ns	ns	ns	ns
Band 4.1/band 3	0.24 ±0.06	0.26 ±0.04	0.18 ±0.04	0,000	0.005	0.24 ±0.04	0.26 ±0.05	0.21 ±0.04	ns	0.02	ns	ns
Band 4.2/band 3	0.14 ±0.02	0.145 ±0.02	0.127 ±0.05	0.007	ns	0.15 ±0.02	0.15 ±0.02	0.12 ±0.02	0.02	0.02	ns	ns
Spectrin/band 3	0.84 ±0.10	0.88 ±0.11	0.63 ±0.11	0.007	0.005	0.87 ±0.11	0.91 ±0.16	0.67 ±0.07	0.005	0.007	ns	ns
Band 5/band 3	0.154 ±0.02	0.158 ±0.01	0.14 ±0.02	ns	ns	0.17 ±0.01	0.16 ±0.02	0.14 ±0.03	0.01	0.007	ns	ns
Tryptophan [arb unit]	483 ±74	448 ±112	203 ±29	0.005	0.005	472 ±55	472 ±97	143 ±46	0.005	0.005	ns	0.009

incubated without t-BOOH and with t-BOOH in concentration 0.1 mmol/L, regardlessly of the fact if the environment included glucose. Only increase of t-BOOH concentration to 1.0 mmol/L led to changes in concentrations of the examined substances. Figure 2 shows proteinogram of erythrocyte membrane. Figure 3 shows densitometric curves. There is dissolving of band 3 protein and low-molecule proteins (below 25 kDa) after incubation of membranes with t-BOOH in concentration of 1.0 mmol/L. These changes are apparent independently of glucose presence in incubation medium. Decrease of spectrin concentration, increase of band 3 protein concentration and the most statistically significant increase of globin concentration ($p<0.001$) are the most distinctive changes. As a result of increased concentration of band 3 protein the proportion between proteins 4.1, 4.2, spectrin, band 5 protein and band 3 protein is decreased.

Tryptophan concentration in membranes decreased. Similarly, as in case of membrane proteins, this change was particularly apparent when t-BOOH in high concentration was used. Decrease of tryptophan concentration

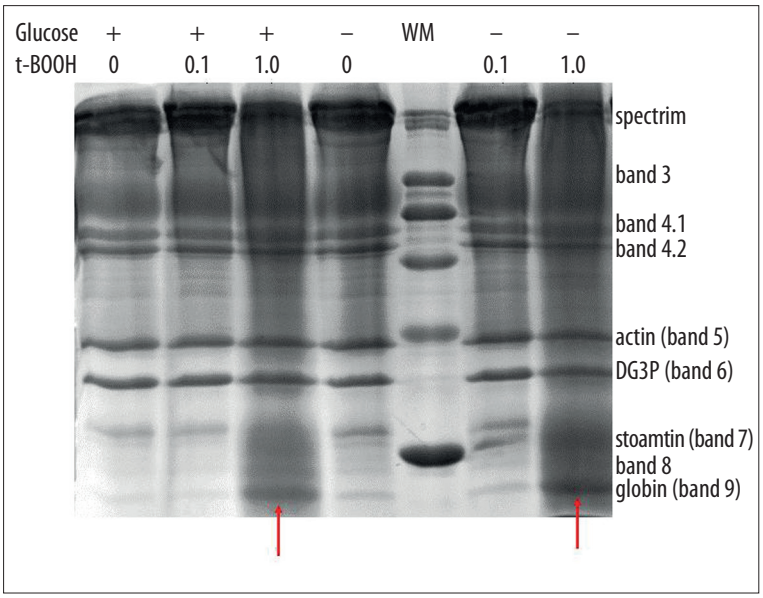

Fig. 2. Electrophoresis of erythrocyte shades after incubation in solutions with glucose and t-butyl hydroperoxide in different concentrations. Arrows indicate the increase of globin amounts

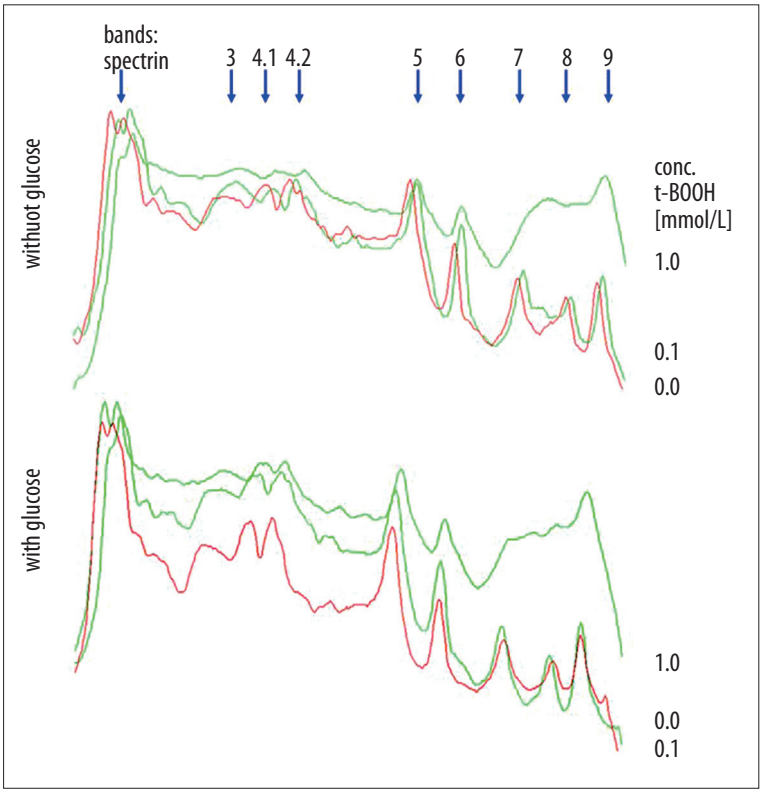

Fig. 3. Densitograms showing protein positions and their changes after exposure to free radicals

(when using t-BOOH in concentration of 1.0 mmol/L) was higher in group with glucose than in group without glucose (p=0.009).

Discussion

Oxidative stress is a state when the production of reactive oxygen species exceeds antioxidant capacities of the defence system.

Tryptophan is one of the indicators of the intensity of oxidative stress. It is an aminoacid present in membrane proteins, especially in spectrin, in which in both units 42 tryptophan residues are present [13]. Tryptophan is one of the factors stabilizing spectrin molecule. Tryptophan molecules are placed in very stable fragments of spectrin, which do not change even if they are exposed to strong denaturating factors, like urea in concentration of 8 mmol/L [14].

In vitro studies confirmed the influence of ROS on tryptophan concentration in membranes. During experiment the use of t-BOOH in high concentration led to decreased concentration of this aminoacid in membranes. This change was more apparent during incubation in medium containing glucose. Changes of tryptophan concentrations were independent of the type of reactive oxygen species. Hydroxyl

radical *in vitro* led to decreased tryptophan concentrations in mitochondrial membranes of rat heart cells [4]. In isolated sarcoplasmic reticulum of the rat exposed to free radicals there were structural changes, accompanied by tryptophan degradation and accumulation of dityrosine, protein and lipid conjugates, conjugated dienes and products of the reaction with thiobarbituric acid. These changes were proportional to the age of rats [4]. Different, interesting results were obtained when mouse mould of chondrosarcoma was exposed to hydrogen peroxide. Low concentrations of ROS led to significant decrease of tryptophan concentrations while high ROS concentrations decreased its concentration only by half [28].

Tryptophan also protects from free radical damage. Transmembrane domains of integral proteins show high condensation of tyrosine and tryptophan, especially in places of high lipid density. It was found that these places play antioxidative role inside lipid bilayer, protecting the cell from oxidative damage. Tyrosine and tryptophan are present in transmembrane fragments of all proteins. Long chains of acyl derivatives of tryptophan and tyrosine are strong inhibitors of lipid peroxidation and death of the cell due to oxidative damage [15]. Significant decrease of tryptophan concentration confirms the loss of protective abilities of erythrocyte membrane.

Guedas et al. found that tryptophan residue in albumin metabolises to kynurenin, hydroxykyrunenin and oxalate under the influence of H_2O_2 [30]. Aneamia was found in patients with chronic inflammation (increased oxidative stress). Its intensity was correlated with the lower concentration of tryptophan and the rise in the concentration of kinurenin in patients' plasma [59]. Also in patients with chronic renal insufficiency the rise of degradation and the decrease in erythrocyte osmotic resistance was observed which shows damage of erythrocyte membrane [55]. Dykens et al. found that products of tryptophan metabolism (mainly 3-hydroxyanthranilate) cause an intensified production of methemoglobin and non-functional oxidation products of hemoglobine. It shows that the process of tryptophan residue degradation occuring under the influence of ROS may intensify oxidative damage of erythrocytes [24].

Glucose is an energetic material necessary in normal erythrocyte metabolism. In physiological conditions glucose is predominantly metabolized in glycolysis. Glucose-6-phosphate is the first product of this process, it is then transformed to 1,3-bisphosphoglycerate (1,3-BPG). Glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) is the last enzyme catalyzing this stage of glycolysis. G-3-PD has specific features: it is a structural component of cytoskeleton [2], on the other hand it is an important enzyme.

The shape of erythrocytes and their ability of deformation are regulated by intracellular cytoskeleton. Electron spectroscopy of paramagnetic resonance performed on erythrocytes incubated with t-BOOH as a source of free radicals [31] showed increased osmotic resistance and decreased mobility of membrane proteins.

Spectrin is cytoskeleton protein responsible for erythrocyte shape, integrity and ability of deformation. ROS lead to decreased spectrin concentration, independently of glucose presence in incubation medium. In the same conditions the concentration of band 3 protein increases. Different results were obtained by Okamoto et al., who found decreased concentration of band 3 protein with simultaneous generation of low-molecule proteins, when influenced by t-BOOH [46]. Different reactions of spectrin and band 3 protein to oxidative stress can be caused by different sources of ROS – free radicals generated outside the cell modify mainly band 3 protein, whereas spectrin is modified by radicals generated inside the cell [12]. ROS generated as a result of hemoglobin oxidation act on spectrin, whereas extracellular oxidants act on band 3 protein. Nevertheless, some reactive forms of oxygen can migrate through membrane to cytoplasm and induce generation of stronger radicals, e.g. ferryl radical. It can cause strong oxidative damage of inner membrane structures.

Erythrocyte membrane is built of lipid bilayer, integral proteins and cytoskeleton. Spectrin is linked with lipid bilayer due to interactions with vertical proteins – band 3 protein and glycophorin C [49]. Ankyrin and band 4.2 protein also take part in vertical links [27,50]. Changes in concentration of these proteins lead to decreased ratio of spectrin to band 3 protein. Similar changes and increased ratio of 4.1 protein and spectrin are present in hereditary spherocytosis [40].

In vitro the concentration of 4.2 protein did not change after incubation in glucose-rich medium, in non-glucose medium the change was small, but statistically significant. Interactions of spectrin with other proteins of cytoskeleton are responsible for horizontal actions. The concentrations of band 4.1 and 4.2 proteins have wide ranges. Incubation of erythrocytes with t-BOOH did not change the amount of actin. Different results in studies *in vitro* were obtained by Caprari et al.[11]. Degradation of spectrin and ankyrin was accompanied by appearance of membrane globins. Simultaneously, the concentration of reduced glutathione decreased. Ultrastructural studies showed that actin molecules form microaggregates causing detachment of actin from spectrin and in this way weakness of cytoskeleton network [11].

We analyzed mutual interactions among proteins in highest amounts: spectrin and band 3 protein, band 4.1, 4.2 and 5 proteins. The proportions between concentrations of these proteins reflect vertical and horizontal interactions stabilizing the erythrocyte structure. It was found that the ratio of proteins 4.1, 4.2, spectrin, band 5 to band 3 protein decreased, what was a consequence of increased concentration of band 3 protein.

When exposed to t-BOOH, the biggest changes in protein amounts were observed in band 8 and 9. Electrophoretic pictures and respective densitograms showed dissolving of individual protein fractions. It was especially apparent for small proteins (below 30 kDa), which in high concentrations appeared after incubation of normal erythrocytes with t-BOOH. Their presence *in vivo* can be the cause of preliminary elimination of erythrocytes from circulation and their half-life shortening.

Many studies show the key role of hemoglobin (or hemichrom) bound to cytoskeleton proteins in the mechanism of erythrocyte aging [33,43,58,57]. Morrison et al. [43] found

hemichroms attached to erythrocyte membrane just before elimination of erythrocyte from circulation. Turrini et al. [57] in studies *in vitro* showed that hemoglobin was bound to the membrane of erythrocytes stimulated with autologic antibodies. Such cells were then phagocytized by macrophages. Probably, the generation of globin and skeletal aggregates in the membrane facilitates the uncovering of hidden antigen places on the outer surface of the cell. As the number of antigen places increases, autological IgG opsonize the cell which dies due to phagocytosis. One of the hypotheses concerning aging of erythrocytes [10] suggests that this process is associated with proteolysis or spatial transformation of membrane proteins caused by cytoskeleton changes.

Young, big erythrocytes have better ability to remove outside the vesicles containing denaturated hemoglobin than old, smaller cells [53]. This effect was confirmed also in conditions *in vitro* [29]. With the aging of erythrocytes the surface of membrane decreases, and in this way the ability to remove vesicles with liposomal products of erythrocyte metabolism and denaturated hemoglobin also decreases. Hemoglobin accumulation in membrane can cause IgG binding and transfer of phosphatydylserine from inner to outer monolayer [35,37,38,39]. Unstable hemoglobin forms, changes in oxidoreductive system in cytoplasm and creation of cross-bond between hemoglobin and cytoskeleton proteins have direct influence on erythrocyte half-life [35,44,49,61]. It was found that small erythrocyte proteins (actin, band 4.1 and 4.2) can take part in creation and release of membrane vesicles, especially during deficiency of main proteins of the membrane [27].

Prooxidative influence of glucose in the conditions of the experiment can be observed in the decreased amount of tryptophan residue of erythrocyte proteins under the influence of ROS in the medium containing glucose when compared to that without it. The presence of glucose does not affect the changes in the amount of particular proteins occuring as a result of ROS influence. It may be caused either by too short exposition time or may be the effect of defence mechanisms. Changes in the membranes of erythrocytes occuring under the influence of ROS may be the reason for shorter half-time of erythrocytes in patients exposed to oxidative stress. This process is intensified in patients with chronic renal disease, especially those receiving hemodialysis, in diabetics and other patients with free radical disease.

Conclusions

In conditions *in vitro* oxidative stress causes damage of cystoskeleton proteins of erythrocytes, present inside the cell and proteins having contact with environment outside the cell. As a consequence, the content of low-molecule proteins increases, mainly globin, which bind to cytoskeleton. This process takes place independently of glucose presence in incubation medium. Aminoacid tryptophan, one of the elements of protein cytoskeleton, also undergoes degradation. Its decrease is higher when erythrocytes are exposed to t-BOOH in environment containing glucose, what can confirm that glucose *in vitro* can exhibit pro-oxidative abilities.

References

[1] An X., Guo X., Sum H., Morrow J., Gratzer W., Mohandas N.: Phosphatidylserine binding sites in erythroid spectrin: location and implications for membrane stability. Biochemistry, 2004; 43: 310–315

[2] Andrade J., Pearce S.T., Zhao H., Barroso M.: Interactions among p22, glyceraldehyde-3 phosphate dehydrogenase and microtubules. Biochem. J., 2004; 384: 327–336

[3] Antonelou M.H., Papassideri I.S., Karababa F.J., Stravopodis D.J., Loutradi A., Margaritis L.H.: Defective organization of the erythroid cell membrane in a novel case of congenital anemia. Blood Cells Mol. Dis., 2003; 30: 43–54

[4] Babusikova E., Jesenak M., Racay P., Dobrota D., Kaplan P.: Oxidative alternations in rat heart homogenate and mitochondria during ageing. Gen. Physiol. Biophys., 2008; 27: 115–120

[5] Bennett V.: The spectrin-actin junction of erythrocyte membrane skeletons. Biochim. Biophys. Acta, 1989; 988: 107–121

[6] Bennett V., Baines A.J.: Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. Physiol. Rev., 2001; 81: 1353–1392

[7] Bober J., Kedzierska K., Safranow K., Kwiatkowska E., Jakubowska K., Herdzik E., Dołegowska B., Domański L., Ciechanowski K.: Influence of glucose in dialyzing fluid on purine concentrations in hemodialyzed patients with chronic renal failure. Nephron. Clin. Pract., 2003; 95: c31–c36

[8] Bober J., Kwiatkowska E., Kedzierska K., Olszewska M., Dołegowska B., Domanski L., Herdzik E., Ciechanowski K., Chlubek D.: Does glucose present in the dialysate limit oxidative stress in patients undergoing regular hemodialysis? Blood Purif., 2005; 23: 219–225

[9] Bober J., Kwiatkowska E., Kedzierska K., Olszewska M., Gołebiewska E., Stachowska E., Kucharska E., Ciechanowski K., Chlubek D.: Influence of glucose in the dialysate on the activity of erythrocyte-glutathione-peroxidase and blood selenium concentration in hemodialyzed patients. Arch. Med. Res., 2007; 38: 330–336

[10] Bratosin D., Mazurier J., Tissier J.P., Estaquier J., Huart J.J., Ameisen J.C., Aminoff D., Montreuil J.: Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. Biochimie, 1998; 80: 173–195

[11] Caprari P., Bozzi A., Malorni W., Bottini A., Iosi F., Santini M.T., Salvati A.M.: Junctional sites of erythrocyte skeletal proteins are specific targets of tert-butylhydroperoxide oxidative damage. Chem. Biol. Interact., 1995; 94: 243–258

[12] Celedón G., González G., Lissi E.A., Hidalgo G.: Free radical-induced protein degradation of erythrocyte membrane is influenced by the localization of radical generation. IUBMB Life, 2001; 51: 377–380

[13] Chakrabarti A., Kelkar D.A., Chattopadhyay A.: Spectrin organization and dynamics: new insights. Biosci. Rep., 2006; 26: 369–386

[14] Chattopadhyay A., Rawat S.S., Kelkar D.A., Ray S., Chakrabarti A.: Organization and dynamics of tryptophan residues in erythroid spectrin: novel structural features of denatured spectrin revealed by the wavelength-selective fluorescence approach. Protein Sci., 2003; 12: 2389–2403

[15] Cibulka R., Racek J.: Metabolic disorders in patients with chronic kidney failure. J. Physiol. Res., 2007; 56: 697–705

[16] Cooke M.S., Henderson P.T., Evans M.D.: Sources of extracellular, oxidatively-modified DNA lesions: implications for their measurement in urine. J. Clin. Biochem. Nutr., 2009; 45: 255–270

[17] Daschner M., Lenhartz H., Bötticher D., Schaefer F., Wollschläger M., Mehls O., Leichsenring M.: Influence of dialisys on plasma lipid peroxidation products and antioxidant levels. Kidney Int., 1996; 50: 1268–1272

[18] Dean R.T., Fu S., Stocker R., Davies M.J.: Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J., 1997; 324: 1–18

[19] Delaunay J.: Molecular basis of red cell membrane disorders. Acta Haematol., 2002; 108: 210–218

[20] Delaunay J., Alloisio N., Morle L., Baklouti F., Dalla Venezia N., Maillet P., Wilmotte R.: Molecular genetics of hereditary elliptocytosis and hereditary spherocytosis. Ann. Genet., 1996; 39: 209–221

[21] DiMascio P., Dewez B., Garcia C.R.: Ghost protein damage by peroxynitrite and its protection by melatonin. Braz. J. Med. Biol. Res., 2000; 33: 11–17

[22] Dodge J. T., Mitchell C., Hanahan D.: The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys., 1963; 100: 119–130

[23] Dołegowska B., Stepniewska J., Ciechanowski K., Safranow K., Millo B., Bober J., Chlubek D.: Does glucose in dialysis fluid protect erythrocytes in patients with chronic renal failure? Blood Purif., 2007; 25: 422–429

[24] Dykens J.A., Sullivan S.G., Stern A.: Glucose metabolism and hemoglobin reactivity in human red blood cells exposed to the tryptophan metabolites 3-hydroxyanthranilate, quinolinate and picolinate. Biochem. Pharmacol., 1989; 38: 1555–1562

[25] Esterbauer H., Schaur R.J., Zollner H.: Chemistry and biology of 4-hydroxynonenal, malonylaldehyde and related aldehydes. Free Radic. Biol. Med., 1991; 11: 81–128

[26] Gallagher P.G.: Red cell membrane disorders. Hematology ASH Education Book, 2005; 1: 13–18

[27] Gallagher P.G.., Ferriera J.D.: Molecular basis of erythrocyte membrane disorders. Curr. Opin. Hematol., 1997; 4: 128–135

[28] Galli F.: Protein damage and inflammation in uraemia and dialysis patients. Nephrol. Dial. Transplant., 2007; 22(Suppl.5): 20–36

[29] Greenwalt T.J., Dumaswala U.J.: Effect of red cell age on vesiculation *in vitro*. Br. J. Haematol., 1988; 68: 465–467

[30] Guedes S., Vitorino R., Domingues R., Amado F., Domingues P.: Oxidation of bovine serum albumin: identification of oxidation products and structural modifications. Rapid Commun. Mass Spectrom., 2009; 23: 2307–2315

[31] Gwoździński K., Janicka M., Brzeszczyńska J., Luciak M.: Changes in red blood cell membrane structure in patients with chronic renal failure Acta Biochim. Pol., 1997; 44: 99–107

[32] Himmelfarb J., McMonagle E., McMenamin E.: Plasma protein thiol oxidation and carbonyl formation in chronic renal failure. Kidney Int., 2000; 58: 2571–2578

[33] Jollow D.J., McMillan D.C.: Oxidative stress, glucose-6-phosphate dehydrogenase and the red cell. Adv. Exp. Med. Biol., 2001; 500: 595–605

[34] Kim K.M., Jung B.H., Paeng K.J., Kim S.W., Chung B.C.: Alteration of plasma total F2-isoprostanes before and after hemodialysis in end-stage renal disease patients. Prostaglandins Leukot. Essent. Fatty Acids, 2004; 70: 475–478

[35] Kuypers F.A., Yuan J., Lewis R.A., Snyder L.M., Kiefer C.R., Bunyaratvej A., Funcharoen S., Ma L., Styles L., de Jong K., Schrier S.L.: Membrane phospholipid asymmetry in human thalassemia. Blood, 1998; 91: 3044–3051

[36] Laemmli U.K.: Cleavage of structural proteins during the assembly of the head of acteriophage T4. Nature, 1970; 227: 680–685

[37] Liu S.C., Yi S.J., Mehta J.R., Nichols P.E., Ballas S.K., Yacono P.W., Golan D.E., Palek J.: Red cell membrane remodeling in sickle cell anemia. Sequestration of membrane lipids and proteins in Heinz bodies. J. Clin. Invest., 1996; 97: 29–36

[38] Low P.S., Rathinavelu P., Harrison M.L.: Regulation of glycolysis via reversible enzyme binding to the membrane protein, band 3. J. Biol. Chem., 1993; 268: 14627–14631

[39] Low P.S., Waugh S.M., Zinke K., Drenckhahn D.: The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. Science, 1985; 227: 531–533

[40] Margetis P., Antonelou M., Karababa F., Loutradi A., Margaritis L., Papassideri I.: Cells physiologically important secondary modifications of red cell membrane in hereditary spherocytosis-evidence for *in vivo* oxidation and lipid rafts protein variations. Blood Cells Mol. Dis., 2007; 38: 210–220

[41] Mazhul V., Shcherbin D., Zavodnik I., Rekawiecka K., Bryszewska M.: The effect of oxidative stress induced by t-butyl hydroperoxide on the structural dynamics of membrane proteins of Chinese hamster fibroblasts. Mol. Cell. Biol. Int., 1999; 23: 345–350

[42] Mohandas N., An X.: New insights into function of red cell membrane proteins and their interaction with spectrin-based membrane skeleton. Transfus. Clin. Biol., 2006; 13: 29–30

[43] Morrison M., Jackson C.W., Mueller T.J., Huang T., Dockter M.E., Walker W.S., Singer J.A., Edwards H.H.: Does cell density correlate with red cell age? Biomed. Biochim. Acta, 1983; 42: S107–S111

[44] Neumann, C.A., Krause D.S., Carman C.V., Das S., Dubey D.P., Abraham J.L., Bronson R.T., Fujiwara Y., Orkin S.H., Van Etten R.A.: Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. Nature, 2003; 424: 561–565

[45] Ohanian V., Wolfe L.C., John K.M., Pinder J.C., Lux S.E., Gratzer W.B.: Analysis of the ternary interaction of the red cell membrane skeletal proteins spectrin, actin, and 4.1. Biochemistry, 1984; 23: 4416–4420

[46] Okamoto K., Maruyama T., Kaji Y., Harada M., Mawatari S., Fujino T., Uyesaka N.: Verapamil prevents impairment in filterability of human erythrocytes exposed to oxidative stress. Jpn. J. Physiol., 2004; 54: 39–46

[47] Palek J., Liu S.C., Snyder L.M.: Metabolic dependence of protein arrangement in human erythrocyte membranes. I. Analysis of spectrin-rich complexes in ATP-depleted red cells. Blood, 1978; 51: 385–395

[48] Pandolfi P.P., Sonati F., Rivi R., Mason P., Grosveld F., Luzzatto L.: Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. EMBO J., 1995; 14: 5209–5215

[49] Reliene R., Mariani M., Zanella A., Reinhart W.H., Ribeiro M.L., del Giudice E.M., Perrotta S., Iolascon A., Eber S., Lutz H.U.: Splenectomy prolongs *in vivo* survival of erythrocytes differently in spectrin/ankyrin- and band 3-deficient hereditary spherocytosis. Blood, 2002; 100: 2208–2215

[50] Rocha S., Rebelo I., Costa E., Catarino C., Belo L., Castro E.M., Cabeda J.M., Barbot J., Quintanilha A., Santos-Silva A.: Protein deficiency balance as a predictor of clinical outcome in hereditary spherocytosis. Eur. J. Haematol., 2005; 74: 374–380

[51] Rogalski A.A., Steck T.L., Waseem A.: Association of glyceraldehyde-3-phosphate dehydrogenase with the plasma membrane of the intact human red blood cell. J. Biol. Chem., 1989; 264: 6438–6446

[52] Rybicki A.C., Heath R., Lubin B., Schwartz R.S.: Human erythrocyte protein 4.1 is a phosphatidylserine binding protein. J. Clin. Invest., 1988; 81: 255–260

[53] Snyder L.M., Fairbanks G., Trainor J., Fortier N.L., Jacobs J.B., Leb L.: Properties and characterization of vesicles released by young and old human red cells. Br. J. Haematol., 1985; 59: 513–522

[54] Srour M.A., Bilto Y.Y., Juma M.: Susceptibility of erythrocytes from non-insulin-dependent diabetes mellitus and hemodialysis patients, cigarette smokers and normal subject to *in vitro* oxidative stress and loss of deformability. Clin. Hemorheol. Microcirc., 2000; 22: 173–180

[55] Tankiewicz A., Pawlak D., Pawlak K., Szewc D., Myśliwiec M., Buczko W.: Anthranilic acid-urae mic toxin damaged red cell's membrane. Int. Urol. Nephrol., 2005; 37: 621–627

[56] Tanner M.J.: Band 3 anion exchanger and its involvement in erythrocyte and kidney disorders. Curr. Opin. Hematol., 2002; 9: 133–139

[57] Turrini F., Arese P., Yuan J., Low P.S.: Clustering of integral membrane proteins of the human erythrocyte membrane stimulates autologous IgG binding, complement deposition, and phagocytosis. J. Biol. Chem., 1991; 266: 23611–23617

[58] Waugh S.M., Walder J.A., Low P.S.: Partial characterization of the copolymerization reaction of erythrocyte membrane band 3 with hemichromes. Biochemistry, 1987; 26:1777–1783

[59] Weiss G., Schroecksnadel K., Mattle V., Winkler C., Konwalinka G., Fuchs D.: Possible role of cytokine-induced tryptophan degradation in anaemia of inflammation. Eur. J. Haematol., 2004; 72:130–134

[60] Yawata Y., Jacob H.S.: Abnormal red cell metabolism in patients with chronic uremia: nature of the defect and its persistencedespite adequate hemodialysis. Blood, 1975; 2: 231–239

[61] Zinkham W.H., Houtchens R.A., Caughey W.S.: Relation between variations in the phenotypic expression of an unstable hemoglobin disorder (hemoglobin Zurich) and carboxyhemoglobin levels. Am. J. Med., 1983; 74: 23–29

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