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The impact of hemodialysis on erythrocyte membrane cytoskeleton proteins

Wpływ hemodializy na białka cytoszkieletu błon erytrocytarnych

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: Hemodialysis (HD) is one of the methods of renal replacement therapy, but it also contributes to an increase in oxidative stress. Hemodialysis leads to changes in the erythrocyte cytoskeleton structure, whilst the presence of glucose in the dialysis fluid which activates the pentose phosphate pathway contributes to the intensification of oxidative stress. Available literature lacks reports on the effect of glucose in the dialytic fluid on the composition of proteins of the cell membrane cytoskeleton.

Material/Methods: Red blood cells for this analysis were collected from patients with chronic renal failure treated with hemodialysis using both glucose-containing and glucose-free dialysis fluid. Following the preparation of membranes, the electrophoretic separation of proteins was performed in denaturing conditions according to Laemmli. The level of tryptophan in membranes was determined by spectrofluorimetry, whilst the activity of glucose-6-phosphate dehydrogenase was determined by measuring the reduction of oxidated NADP.

Results: Hemodialysis in both groups of patients resulted in a statistically significant reduction of tryptophan as an oxidative stress indicator when compared to the control group. Moreover, the activity of glucose-6-phosphate dehydrogenase in the group of patients was higher than in the control group, and following the HD procedure it decreased, which may have been caused by a reduced concentration of dialyzed glucose. The HD procedure affects the structure of the erythrocyte membrane cytoskeleton, which is reflected in the concentration changes in individual proteins and in their mutual relationships corresponding to vertical and horizontal interactions stabilizing the structure of the erythrocyte membrane cytoskeleton. These changes may contribute to the shortening of cell lifespan.

Keywords: oxidative stress • hemodialysis • erythrocytes • cytoskeleton proteins

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Abbreviations: **CRF** – chronic renal failure, **G3P-DH** – 3-phosphoglycerol aldehyde dehydrogenase, **G6P-DH** – glucose-6-phosphate dehydrogenase, **PBS** – buffered solution of sodium chloride, **PPP** – pentose phosphate pathway

Introduction

The ischemia that commonly accompanies renal diseases has a significant impact on the quality of life in chronic renal failure (CRF) patients. An important cause of ischemia is the shortened lifespan of erythrocytes due to increased hemolysis. Besides underlying diseases, the background of the disorder includes e.g. oxidative stress. Our earlier studies focused on the effects of glucose contained in the dialytic fluid on the erythrocyte antioxidant system in hemodialyzed CRF patients. Glucose contained in the dialytic fluid impacts the glutathione antioxidant system. It activates the pentose phosphate pathway (PPP), leading to production of reducing equivalents required e.g. for the recovery of reduced glutathione [4,5] and changing the energy status of the blood cell [4,41]. Although glucose enhances oxidative stress, it also reduces the cell's capability to undergo hemolysis [16]. Available literature lacks reports on the effect of glucose contained in the dialytic fluid on composition of erythrocyte cytoskeletal proteins. Ischemia in CRF patients is associated with enhanced glycolysis and accumulation of high energy compounds within the erythrocytes. Ischemia in CRF patients was found to be associated with increased erythrocytic consumption of glucose [39,45].

Erythrocyte cell membrane

Erythrocytes live for an average of 120 days and are very well adapted to their tasks by containing a very viscous "liquid crystal" – hemoglobin – encapsulated in a protein skeleton with lipid bilayer. Cytoskeletal proteins are highly differentiated, including e.g. transport protein as well as enzymatic proteins. The main transport protein is band 3 anion transport protein which, besides exchanging anions, acts as a binding site between the membrane skeleton and the erythrocyte membrane. It is also the main location for the binding of hemichromes and hemoglobin to the cell membrane. The N-terminal domain of band 3 is anchored within the cytoplasm by interactions with ankyrin and proteins 4.1 and 4.2.

The main components of membrane skeleton include spectrin, actin, band 4.1 protein, ankyrin and adducin [28]. Spectrin, the most important cytoskeletal protein, is a heterodimer consisting of alpha and beta chains combined with actin to form a net lining the cytoplasmic membrane surface. It also binds ankyrin and band 4.1 protein [1]. Band protein 5 binds actin as well as spectrin and band 4.1 protein, which is also bound to spectrin. These three proteins stabilize the horizontal structure of the cytoskeleton. Band 4.2 protein is also bound to band 3 protein, although its role has not been unambiguously determined. The most important fraction of band 7 protein is stomatin (7.2), deficiency of which leads to stomatocytosis – a disorder associated with excessive permeability of the cell membrane [14,17,29]. The increase in band 7 protein is associated with an increase in the levels of membrane-bound globin in ischemic patients [3].

3-Phosphoglycerol aldehyde dehydrogenase (G3P-DH) (band 6), a glycolytic enzyme catalyzing transformation of 3-phosphoglycerol aldehyde into 1,3-bisphosphoglycerate, is one of the three erythrocyte membrane proteins with enzymatic activity. G3P-DH is bound to the cytoplasmic domain of band 3 protein [38].

Band 9 protein – globin – is formed by degradation of hemoglobin and bound to erythrocyte membrane cytoskeleton, most typically to spectrin. The levels of globin increase upon echinocytic transformation of erythrocytes and oxidative stress, as well as in hereditary spherocytosis [27].

Changes in erythrocytes, manifested mainly as echinocytosis, are often observed in hemodialyzed patients. These changes are not dependent on the levels of uremic toxins and are rare in CRF patients undergoing conservative treatment [44]. Main changes in the erythrocyte cytoskeleton structure include reduction in spectrin levels and increase in ankyrin levels. Also changed are the proportions between individual proteins, leading to horizontal and vertical disturbances in the membrane structure [12,13].

Selected aspects of erythrocyte metabolism

Upon oxidative stress experienced during dialysis, erythrocytes consume 1.7 times the amount of glucose consumed in physiological conditions. Following incorporation into erythrocytes, glucose is phosphorylated into glucose-6-phosphate, which is then metabolized by glycolysis or by means of the pentose phosphate pathway (PPP). Glycolysis is the main source of energy for erythrocytes, while the PPP pathway is the only erythrocytic source of NADPH. In physiological conditions, only several percent of glucose-6-phosphate are metabolized by the PPP pathway; however, the consumption of this enzyme is increased as much as 20 times upon oxidative stress that increases the demand for its activity [33]. The activity of the PPP is regulated by the first enzyme involved in the pathway, i.e. glucose-6-phosphate dehy-

drogenase (G6P-DH). Oxidative stress is one of the causes of changes in G6P-DH activity [47]. Due to numerous antioxidant systems included in their cytoplasm, erythrocytes are considered to be scavengers of free radicals. However, they also include an intraerythrocytic source of reactive oxygen species, i.e. hemoglobin. Erythrocytic concentration of hemoglobin is ca 5. mmol/L, while concentration of heme iron is about 20 mmol/L. Erythrocytic cytoplasm is rich in oxygen which, in the presence of e.g. heme iron, is transformed into reactive species, mostly free radicals. This leads to formation of methemoglobin and a superoxide anion radical which attacks thiol groups within hemoglobin molecules and cytoskeletal proteins. As a result, protein aggregates are formed, consisting of hemoglobin moieties and cytoskeletal proteins linked by disulfide bonds [20].

Reduced membrane tryptophan content is one of the markers of oxidative stress intensification. Tryptophan is an amino acid abundantly present in membrane proteins, particularly spectrin [8], and stabilizing their molecules. By degrading proteins, reactive oxygen species eliminate tryptophan moieties from cell membranes [9].

Material and methods

Patients

The study population consisted of 64 patients enrolled in three groups: patients with chronic renal failure hemodialyzed with glucose-free dialytic fluids (HD-g(–), n=21), patients with chronic renal failure hemodialyzed with glucose-containing dialytic fluids (HD-g(+), n=22), and the control group (K, n=21). Patients were under medical care at the Clinic of Nephrology, Transplantology and Internal Diseases of the Pomeranian Medical University. All subjects agreed to take part in the study. The study was granted approval by the Bioethics Committee at the Pomeranian Medical University.

Patients were hemodialyzed 3 times a week, 4 hours per dialysis. Fresenius dialyzers with polysulfate dialytic membranes were used. Composition of the dialytic fluid (following reconstitution of concentrate) was as follows: 138 mmol/L Na^+; 0, 2.0 or 3.0 mmol/L K^+; 1.75 mmol/L Ca^{2+}; 0.5 mmol/L Mg^{2+}; 107.5 mmol/L Cl^-; 0 or 5.6 mmol/L glucose, 32 mmol/L HCO_3^-. None of the subject underwent red concentrate cells (RCC) or received iron products within 4 weeks before the study.

The adequacy of hemodialysis was determined by the Kt/V ratio. The value of Kt/V ranged from 1.0 to 1.2. All patients received subcutaneous recombinant human erythropoietin (NeoRecormon). Chronic renal failure was caused by: chronic glomerulonephritis (16 patients), chronic pyelonephritis (10 patients), or autosomal dominant polycystic kidney disease (9 patients). In 8 patients, CRF was either due to other causes or no cause had been determined.

Patients qualified for the study were free of diabetes and active cancer and suffered no active inflammation as confirmed by the assessment of CRP levels during the study. The mean age of subjects in the control group was not statistically different from that in the study groups. Subjects in the control group had no history of diseases linked to a free radical background and were free of active cancer or active inflammation.

Methods

Blood for analyses was collected from hemodialyzed patients immediately before and immediately after hemodialysis. Due to different times of dialysis start, the long duration of dialyses and higher risk of hypoglycemia during HD, patients were not fasting upon blood collection. In the control group, blood was collected in the morning from fasting patients. Erythrocyte membranes were prepared according to Dodge's procedure [15]; protein separation was achieved on polyacrylamide gel in denaturing conditions according to Laemmli [22]. Qualitative analysis of densitograms was performed using GelScan software (Kucharczyk TE). Membrane tryptophan levels were determined by spectrofluorimetry [35], while the activity of glucose-6-phosphate dehydrogenase was measured by determination of oxidated NADP ($NADP^+$) [36].

Statistical analysis

After determination that distribution of measurable values was not normal (Shapiro-Wilk test), the following non-parametric tests were used: Wilcoxon's test for paired samples to examine differences in parameters before and after HD, the Mann-Whitney U-test to determine differences between groups, and Spearman's test to determine correlations between the assessed parameters. The analysis was performed using Statistica 10.0 software (StatSoft Krakow).

Results

Table 1 presents the characteristics and basic laboratory parameters of individual study groups.

Higher values of erythrocytic indices, mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV), and lower red cell distribution width (RDW) values were observed in patients dialyzed with glucose-containing fluid as compared to the other group of CRF patients.

The contents of separated proteins in the erythrocytes membranes in dialysis groups and in control shown in Table 2.

The content of tryptophan in erythrocyte membranes of dialyzed patients in both groups was lower than that in the control group ($p<0.001$). Hemodialysis and composition of dialytic fluid had no effect on tryptophan levels.

The activity of G6P-DH in both groups of CRF patients was higher than that in the control group. Hemodialysis led to a statistically significant reduction in the activity of the enzyme in the HD-g(+) group.

Following electrophoretic separation of erythrocyte membrane proteins, the following proteins were subjected to analysis: spectrins (alpha and beta together), band 3, 4.1, 4.2, 5 (actin), 6 (3-phosphoglyceraldehyde dehydrogenase), 7, and 8 proteins and globin. The levels of spectrins in erythrocytes of tested patients were markedly lower than in the control group. No significant changes occurred as the result of hemodialysis. Concentration of band 3 protein was higher than in the control group both before and after HD in both HD-g(–) and HD-g(+) groups. Band 4.1 protein levels were similar in all study groups and were characterized by a wide distribution of values, similar as in the case of band 4.2 protein levels. The HD-g(–) group was characterized by lower levels of band 4.2 protein (before HD) compared to the remaining groups. Proteins of bands 6, 7, 8 and 9 (globin) were present in higher amounts in both CRF patient groups than in the control group.

Besides the levels of individual proteins, the analysis also included their quantitative ratios responsible for vertical and horizontal connections. Hemodialysis led to a significant increase in the ratio between band 4.1 protein and spectrin levels in HD-g(+) group as well as in the ratio between actin and band 3 protein levels in both CRF patient groups. When comparing concentration ratios between the groups of dialyzed patients, differences were observed with regard to the ratio of pre-HD band 4.1 and band 3 protein levels, which was higher in the HD-g(+) group, as well as to the ratio of post-HD actin and band 3 protein levels, which was also higher in the HD-g(+) group.

Similarly as in the analysis of individual proteins, large differences were observed between the dialyzed groups and the control group. The ratio of concentrations of band 4.1 and band 3 proteins was lower than in the control group in both CRF patient groups before the dialysis as well as after the dialysis in the HD-g(–) group. Similarly, the ratio of concentrations of band 4.2 and band 3 proteins before the dialysis was lower in both CRF patient groups than in the control group.

The ratio of concentrations of actin (band 5) to band 3 protein is of particular note. Compared to the control group, the ratio was higher in both dialyzed groups before HD and lower after HD. Very large, statistically significant differences were observed between the ratios of concentrations of spectrin and band 3 protein for both groups of dialyzed patients compared to the control group. The ratios were always lower in dialyzed patients, both before and after HD.

Fig. 1 presents the selected protein profiles in individual study groups. The image of separated proteins from healthy individuals consists of clearly differentiated, easily identifiable bands corresponding to individual proteins. Protein profiles in CRF patients are more diffuse. Particular difficulty is encountered when attempting to unanimously ascribe bands to low molecular proteins (< 30 kDa) and band 3 protein. The images before and after hemodialysis are very similar. Although any single hemolytic procedure leads to virtually no changes in protein profiles, a significant reduction in spectrin levels could be observed following hemodialysis in some patients.

Correlations between examined parameters

A positive correlation was observed between the activity of G6P-DH and cell membrane tryptophan levels in the HD-g(+) group after HD (R_s=+0.566, p=0.007) (Fig. 2).

All correlations described below pertain to pre-dialysis conditions. In the HD-g(+) group, positive correlations were observed between the levels of band 4.1 protein and tryptophan (R_s=+0.584, p=0.004), between the ratio of levels of band 4.1 and spectrin and tryptophan levels (R_s=+0.540, p=.009) between the ratio of levels of band 4.1 and band 3 proteins and tryptophan levels (R_s=+0.543, p=0.009). The band 6 protein level was negatively correlated with globin levels (R=–0.614, p=0.002).

Negative correlations between globin and spectrin levels were observed in both hemodialyzed groups (Fig. 3). A similar correlation was observed between globin levels and the ratios of levels of spectrin and band 3 protein. In the HD-g(+) group, the correlation coefficient was R_s= –0.519, p=0.016; the correlation was particularly strong in the HD-g(–) group: R_s= –0.799, p<0.001.

Discussion

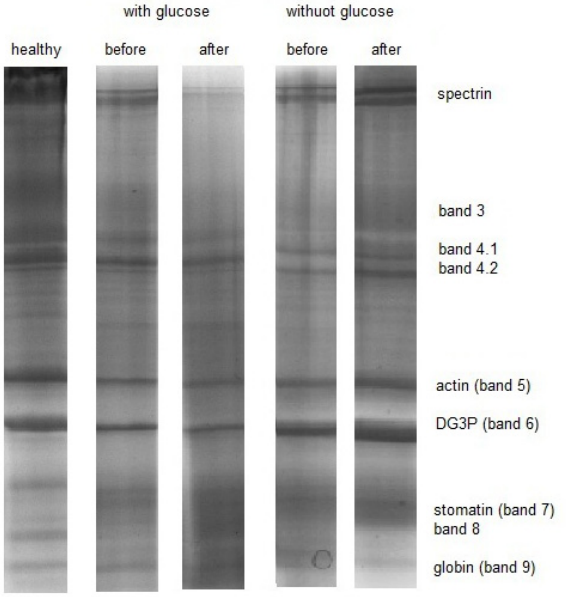

Fig. 1. Protein profile of selected erythrocyte membranes

Table 1. Characteristics and hematological parameters of blood in study groups.

Parameter	Dialyzed patients		Control group
	without glucose	with glucose	
Glucose [mmol/L] (before HD)	4.0 ± 1.49	4.36 ± 1.36	4.46 ± 0.16
Glucose [mmol/L] (after HD)	3.82 ± 1.81	4.87 ± 1.40	
Sex (F/M)	10/11	10/12	10/11
Age [years]	54.6 ± 9.5	58.3 ± 11.5	55.8 ± 7.1
Time from first dialysis [months]	28.2 ± 12.6	26.9 ± 14.50	
WBC [G/L]	7.69 ± 1.93	6.59 ± 2.09	6.23 ± 1.67
Hemoglobin [mmol/L]	6.03 ± 0.75	6.07 ±0.60	8.76 ± 0.57
Hematocrit [L/L]	31.4 ± 4.4	29.2 ± 2.96	41.9 ± 2.97
RBC [T/L]	3.57 ± 0.46	3.24 ± 0.38	4.75 ± 0.30
MCHC [mmol/L]	19.59 ± 0.92	20.78 ± 0.41*	20.82 ± 0.26
MCH [fmol]	1.73 ± 0.16	1.86 ± 0.10*	1.84 ± 0.06
MCV [fL]	88.29 ± 5.86	89.79 ± 3.29	88.63 ± 2.40
RDW [%]	15.67 ± 1.22	15.30 ± 1.17*	14.11 ± 0.89
Urea [mmol/L] (before HD)	50.41 ± 14.76	46.51 ± 16.81	5.78 ± 0.70
Urea [mmol/L] (after HD)	9.12 ± 2.24	7.07 ± 2.15	
Creatinine [mmol/L] (before HD)	806 ± 198	625 ± 190	93.7 ± 12.38
Creatinine [mmol/L] (after HD)	374 ± 112	481 ± 175	

* – statistically significant differences between groups dialyzed with glucose-containing and glucose-free fluids

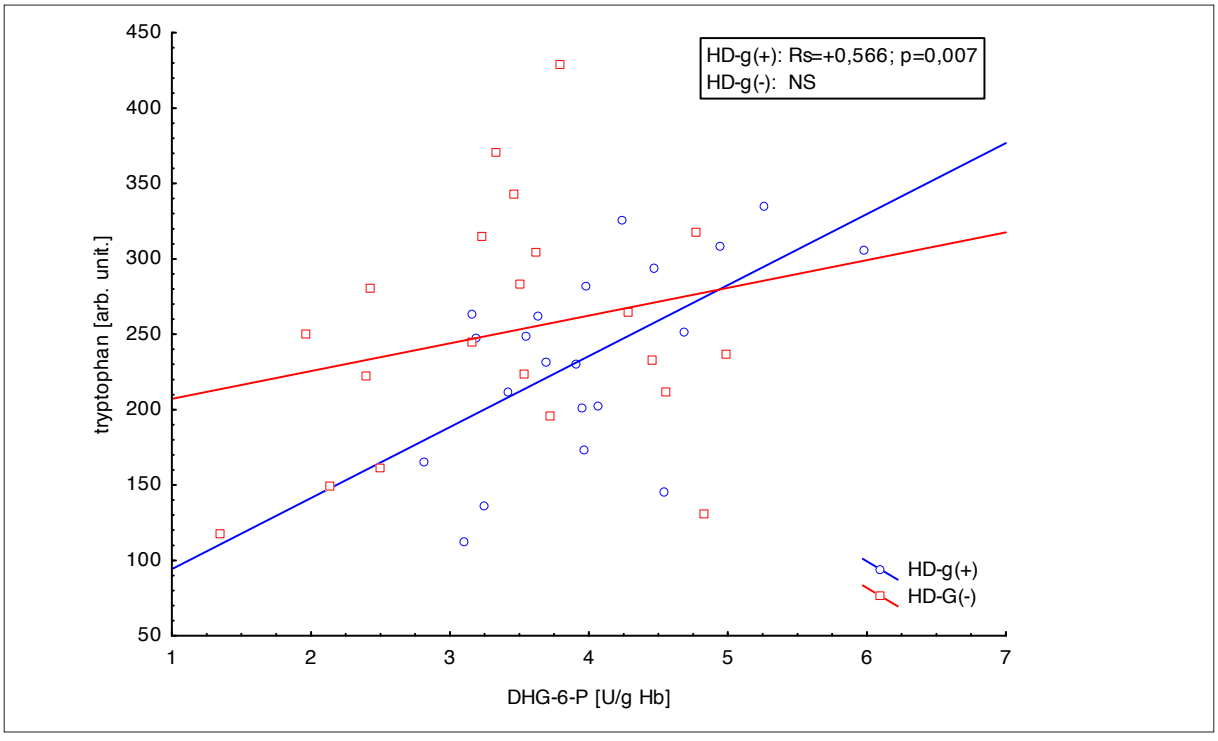

Fig. 2. Correlation between tryptophan levels and G6P DH activity after HD

Table 2. Erythrocyte membrane proteins.

Parameter	Time of measurement	HD-g(+)	HD-g(–)	K	K vs HD-g(+)	K vs HD-g(–)
spectrin [mg/100 mg prot.]	before HD	23.88 ± 2.37	23.32 ± 3.61	30.84 ± 2.37	<0.001	<0.001
	after HD	23.59 ± 2.59	23.98 ± 3.03		<0.001	<0.001
band 3 [mg/100 mg prot.]	before HD	38.47 ± 2.87	41.34 ± 3.02‡	36.55 ± 2.03	0.02	<0.001
	after HD	38.34 ± 3.42	40.69 ± 2.21‡		0.02	<0.001
band 4.1 [mg/100 mg prot.]	before HD	7.38 ± 1.42	7.96 ± 1.80	8.57 ± 1.50	0.008	
	after HD	8.40 ± 2.33	7.74 ± 2.30			
band 4.2 [mg/100 mg prot.]	before HD	4.77 ± 0.94	4.18 ± 0.74‡	5.18 ± 0.69		<0.001
	after HD	5.17 ± 1.10	4.81 ± 1.45			
band 5 [mg/100 mg prot.] (actin)	Before HD	5.10 ± 1.05	5.43 ± 0.81*	5.88 ± 0.52	0.005	0.03
	after HD	5.18 ± 1.09	6.03 ± 0.90		0.01	
band 6 [mg/100 mg prot.]	before HD	7.13 ± 1.70	6.26 ± 1.00	5.52 ± 0.88	0.002	0.05
	after HD	7.09 ± 1.63	6.44 ± 0.83		<0.001	0.003
band 7 [mg/100 mg prot.]	before HD	4.87 ± 1.57	3.94 ± 1.51‡	3.25 ± 0.71	<0.001	
	after HD	4.17 ± 0.81	3.50 ± 1.32‡		0.001	
band 8 [mg/100 mg prot.]	before HD	3.90 ± 1.34	3.62 ± 1.35	1.93 ± 0.53	<0.001	<0.001
	after HD	3.87 ± 0.87	3.26 ± 0.96‡		<0.001	<0.001
band 9 [mg/100 mg prot.] (globin)	before HD	4.65 ± 1.16	3.95 ± 1.50	2.39 ± 0.67	<0.001	<0.001
	after HD	4.39 ± 1.81	3.57 ± 1.30‡		<0.001	0.001
band 4.1/band 3	before HD	0.21 ± 0.08	0.19 ± 0.05	0.24 ± 0.05	0.009	0.004
	after HD	0.24 ± 0.10	0.19 ± 0.06			0.006
band 4.1/spectrin	before HD	0.34 ± 0.13*	0.36 ± 0.12	0.28 ± 0.05		
	after HD	0.42 ± 0.18	0.33 ± 0.13		0.003	
band 4.2/band 3	before HD	0.13 ± 0.03	0.10 ± 0.02‡	0.14 ± 0.02	0.03	<0.001
	after HD	0.14 ± 0.03	0.12 ± 0.03			0.005
band 5/band 3	before HD	0.21 ± 0.04*	0.23 ± 0.05*	0.19 ± 0.02	0.03	0.006
	after HD	0.14 ± 0.03	0.15 ± 0.03‡		<0.001	<0.001
spectrin/band 3	before HD	0.62 ± 0.09	0.57 ± 0.12	0.85 ± 0.10	<0.001	<0.001
	after HD	0.59 ± 0.11	0.59 ± 0.10		<0.001	<0.001
DH G-6-P [U/g Hb]	before HD	4.42 ± 0.85	3.72 ± 0.69‡	2.52 ± 0.71	<0.001	<0.001
	after HD	3.99 ± 0.78*	3.43 ± 1.02		<0.001	0.003
tryptophan [arb. unit]	before HD	234.4 ± 62.1	251.9 ± 79.2	485.3 ± 63.2	<0.001	<0.001
	after HD	221.3 ± 60.4	242.7 ± 95.6		<0.001	<0.001

Values reported as means ± SD. Statistically significant differences for values after HD as compared to values before HD are marked with * – $p<0.05$. Statistically significant differences ($p<0.01$) between HD-g(+) vs HD-g(–) groups are marked with ‡

Oxidative stress that accompanies CRF intensifies disorders such as diabetes, uncontrolled hypertension and autoimmune diseases which are often associated with increased production of reactive oxygen species; these diseases are not associated with CRF itself.

A statistically significant reduction of the levels of tryptophan – an oxidative stress marker – was observed in both patient groups as compared to the control group. Among its other functions, tryptophan protects cells from free radical damage. Transmembrane domains of membrane integrated proteins are characterized by high concentrations of tryptophan, particularly at sites with a high density of lipids. The sites were found to play an antioxidative role within the lipid bilayer, protecting the cells from oxidative damage Long chains of acylated

derivatives of tryptophan and tyrosine are strong inhibitors of lipid peroxidation and apoptosis due to oxidative damage [11]. The significant reduction in tryptophan levels in the study groups suggests both high oxidative stress to the blood cells and the loss of the protective properties of erythrocyte membranes.

Glucose is the energy source essential for correct metabolism within the erythrocytes. As a low-molecular substance it "escapes" the HD process, and its deficits are not sufficiently fast replenished by gluconeogenesis. In physiological conditions, glucose is largely metabolized in the process of glycolysis. The first product in this process is glucose-6-phosphate, which is transformed into 1,3-bisphosphoglycerate (1,3-BPG) in a series of reactions. The last enzyme catalyzing this fragment of glycolysis is 3-phosphoglycerol aldehyde dehydrogenase (G3P-DH). It has very specific properties: on one hand it is a cytoskeletal building block [2], and on the other it is an enzyme. Changes in G3P-DH in erythrocyte membranes of dialyzed patients are considered in terms of band 6 protein as a building block; an increase in band 6 protein levels is associated with changes in interactions between membrane proteins and loss of membrane stability [12]. Similarly as previously reported by Costa et al. [12,13], patients had significantly elevated levels of G3P-DH compared to the control group. Concentrations of band 6 protein were higher in both CRF patient groups as compared to the control group both before and after hemodialysis. In the HD-g(–) group, concentration of band 6 protein increased slightly upon HD. Higher mean values observed in the HD-g(+) group as compared to the HD-g(–) group and the control group suggest that glucose plays a role in the erythrocytic metabolism during the dialysis.

When analyzing the results obtained in this study, one should look at G3P-DH in terms of its enzymatic activity. The bound enzyme is inactive and plays the role of a functional reserve [24] activated upon oxidative stress [26]. Harrison [18] observed that oxidants stimulate phosphorylation of band 3 protein binding sites, releasing G3P-DH and activating glycolysis within erythrocytes. Increased production of 1,3-BPG occurs as the substrate glucose is present at higher concentrations than in the HD-g(–) group. 1,3-BPG may undergo transformation to 2,3-bisphosphoglycerate, catalyzed by bisphosphoglycerate mutase. In CRF patients, 2,3-BPG levels rise along with the rise in ATP levels in erythrocytes. High 2,3-BPG levels allow a reduction of hemoglobin's affinity to oxygen, providing better oxygen supply to the tissues. This might be a defense mechanism protecting CRF patients from the consequences of ischemia.

Upon oxidative stress experienced during dialysis, erythrocytes consume a much higher amount of glucose than in physiological conditions. This is probably due to significant activation of the pentose phosphate pathway. The activity of the PPP is regulated by the first enzyme involved in the pathway, i.e. glucose-6-phosphate dehydrogenase (G6P-DH). The activity of G6P-DH in both groups of patients was markedly higher than in the control

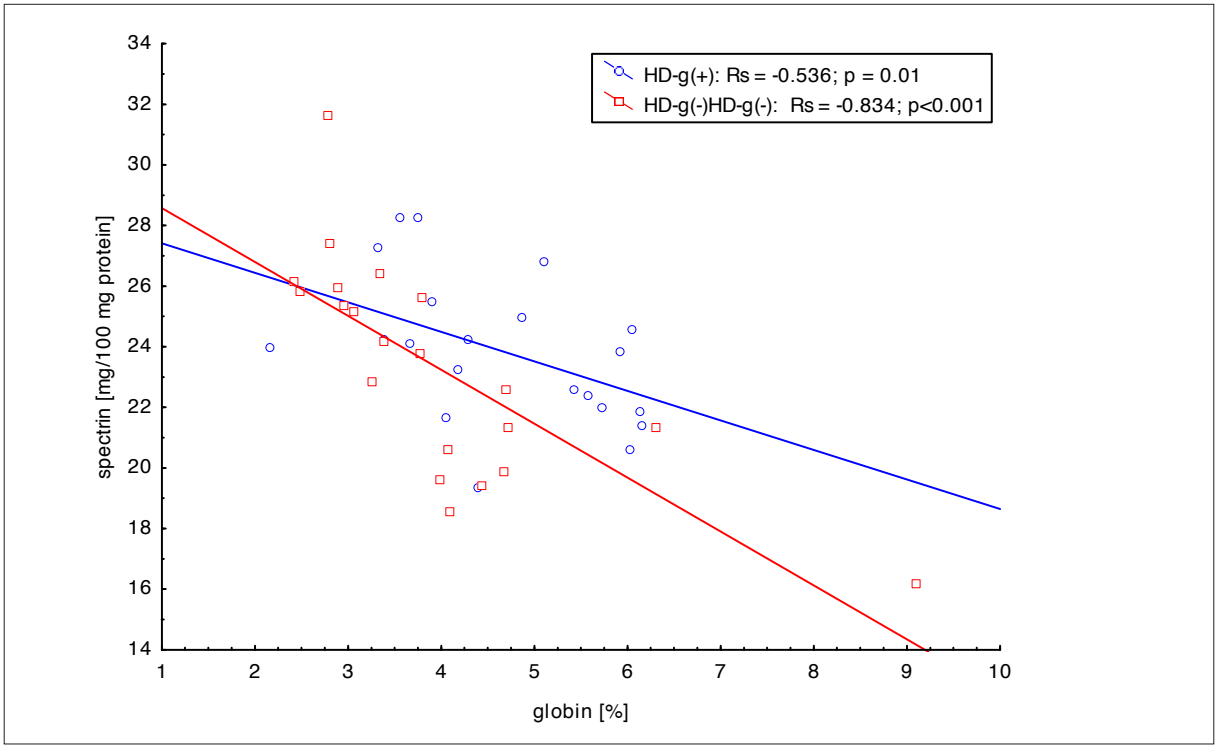

Fig. 3. Correlation between spectrin and globin levels

groups. Hemodialysis reduces the activity of the enzyme in both groups, which might be due to a reduction in concentrations of dialyzed glucose in patients' blood. The higher activity of G6P-DH in the HD-g(+) group was due to the higher levels of glucose, which was not dialyzed out of the circulation fast enough due to its presence in the dialytic fluid. The higher activity of G6P-DH is one of the factors reducing oxidative stress. Denaturation of hemoglobin in oxidative stress conditions leads to heme release and binding to the erythrocyte membrane. Deformability of erythrocytes with G6P-DH deficiency is drastically reduced in the presence of hydroxyl radicals [10]. The strongly positive correlation between the activity of G6P-DH and tryptophan levels in the membranes of patients in the HD-g(+) group confirms the role of G6P-DH in antioxidative defense. This correlation was not observed in the HD-g(–) group, where blood glucose levels during the HD procedure are low, or in the control group, where no oxidative stress was experienced.

Erythrocytic changes independent of uremic toxin levels, manifested mainly as echinocytosis, were observed in hemodialyzed CRF more frequently than in patients undergoing conservative treatment [44]. Consequences of hemodialysis include e.g. formation of non-normal biological complexes leading to changes in erythrocyte structure. Osmotic resistance of erythrocytes is changed [19].

Spectrin is the main cytoskeleton protein responsible for the shape, integrity and deformability of erythrocytes. The cytoskeleton is bound to the lipid bilayer by vertical connections of spectrin, band 3 protein and glycophorin [37,42]. The levels of spectrins in erythrocytes of tested patients was decidedly lower in both HD-g(+) and HD-g(–) groups than in the control group. Similar results were obtained in patients dialyzed using polysulfate dialyzers; however, no dialytic fluid composition was presented in the article [12]. The quantity of spectrin in the HD-g(+) group was directly proportional to the quantity of tryptophan within the membrane. This correlation was not observed in the other patient group.

Concentration of band 3 proteins in the erythrocytes of study patients was higher than in the control group. Although hemodialysis did not significantly change the levels of this protein between both patient groups, reduction in its concentration was observed, particularly in the HD-g(–) group. Different results, i.e. reduction in the levels of band 3 protein, were observed by others [12]. Opposite reactions of spectrin and band 3 proteins to the oxidative stress may be due to different sources of reactive oxygen species (ROS) – free radicals generated extracellularly affect mainly band 3 protein, while spectrin is modified by radicals generated within the cell (e.g. oxidation of hemoglobin) [7].

Erythrocyte membrane consists of a lipid bilayer, integral membrane proteins and cytoskeleton. Spectrin is bound to the lipid bilayer by interactions with vertical proteins – band 3 and glycophorin C [34]. Vertical connections also involve ankyrin and band 4.2 protein [17,37]. Due to the changes in concentrations of these proteins, the ratio of spectrin and band 3 protein levels is also reduced. Similar disturbances in the proportions as well as an increase in the ratio of band 4.1 and spectrin are observed in hereditary spherocytosis [27].

Band 4.2 protein levels are characterized by wide distribution of results in all study groups. However, the pre-HD levels in the HD-g(–) group were statistically significantly lower than in groups K and HD-g(+). No difference was observed in post-HD levels. The pre-HD ratio of concentrations of band 4.2 and band 3 proteins, responsible for interactions between integral and cytoskeleton proteins, was lower in both CRF patient groups than in the control group. These results are consistent with literature data [12,13].

Connections of spectrin and cytoskeletal proteins other than those discussed above are responsible for horizontal interactions.

The levels of band 4.1 protein, similarly to band 4.2 protein, are characterized by wide distribution and are similar in all study groups. The only statistically significant difference was observed between levels in the control group and the pre-HD levels in the HD-g(+) group. Hemodialysis had no effect on band 4.1 protein levels. No statistically significant differences were observed between the two CRF patient groups.

The pre-HD levels of band 5 protein, i.e. actin, in erythrocyte membranes of dialyzed patients in both groups were lower than in the control group. Hemodialysis leads to an increase in band 5 protein levels in the HD-g(–) group. No differences were observed between HD-g(+) and HD-g(–) groups after HD, while a statistically significant difference between groups K and HD-g(–) was maintained.

Band 7 protein is very heterogeneous. Elevated levels of this protein were observed in both groups of hemodialyzed CRF patients. Margetis et al. observed increased levels of band 6 and 7 proteins, an increased band 4.1/spectrin ratio, and a reduced spectrin/band 3 protein ratio in hereditary spherocytosis. The increase in protein levels may reflect changes in interactions between membrane proteins and destabilization of the membrane structure [27].

Mutual relationships between the most abundant proteins, i.e. spectrin and band 3 proteins and band 4.1, 4.2 and 5 proteins, were analyzed. The relationships between the levels of these proteins correspond to vertical and horizontal interactions stabilizing the erythrocyte structure. Hemodialysis leads to a significant increase in the ratio between band 4.1 protein and spectrin levels in HD-g(+) as well as in the ratio between actin and band 3 protein levels in both protein groups.

When comparing concentration ratios between the groups of dialyzed patients, differences were observed with regard to the ratio of pre-HD band 4.1 and band 3 protein levels, which was higher in the HD-g(+) group, as well as to the ratio of post-HD band 5 to band 3 protein levels, which was also higher in the HD-g(+) group. The ratio of concentrations of band 4.1 and band 3 proteins was lower than in the control group in both CRF patient groups before the dialysis as well as after the dialysis in the HD-g(–) group.

Costa et al. [12] observed significant changes in membrane protein composition in dialyzed patients: the increased ratio of ankyrin/band 3 protein and spectrin/ankyrin levels as well as the increased ratio of spectrin and band 4.1 levels due to reduced spectrin levels and an increased ratio of spectrin and band 3 levels.

Numerous studies suggest the key role of hemoglobin (or hemichrome) binding to cytoskeletal proteins in the mechanism of erythrocyte aging [20,30,43,46]. Morrison et al. [30] detected hemichromes bound to erythrocyte membrane just before elimination of blood cells from the circulation. Turrini et al. [43] demonstrated that hemoglobin binds the membrane of erythrocytes stimulated with autologous antibodies *in vitro* and that thus obtained blood cells undergo phagocytosis by macrophages. Probably, formation of aggregates of globin and skeletal proteins (e.g. band 3 proteins) within the membrane facilitates exposure of hidden antigen sites on the outer cell surface. As the number of antigen sites increases, cells are opsonized by autologous circulating IgGs and undergo cell death due to phagocytosis. One of the hypotheses regarding erythrocyte aging [6] assumes that the process is due to proteolysis or spatial reconstruction of membrane proteins as a result of cytoskeletal changes.

Young, large erythrocytes are more capable of expelling vesicles containing denatured hemoglobin than older and smaller cells [40]. As erythrocytes age, the surface of the membrane is reduced; this is associated with reduction of the ability to eliminate erythrocyte metabolic products, including denatured hemoglobin, with liposomal vesicles. Accumulation of denatured hemoglobin within the cellular membrane may cause binding of the IgGs and transport of phosphatidylserine from the inner monolayer to the outer monolayer [21,23,24,25]. Unstable forms of hemoglobin, changes in the cytoplasmic oxidative/reductive system, and formation of cross-linkages between hemoglobin and cytoskeletal proteins directly affect erythrocyte survival [21,31,34,48]. Smaller erythrocytic proteins (actin, band 4.1 and 4.2) may play a role in formation and release of membrane vesicles, particularly upon concomitant deficiencies in main membrane proteins [34].

An inverse relationship was observed between the levels of spectrins and globins in both study groups. Although the relationship was weak in the HD-g(+) group, it was very strong in the HD-g(–) group. Only in the HD-g(+) group were the levels of band 6 protein (G6P-DH) negatively correlated with the levels of band 8 protein and globins. These relationships may suggest a protective effect of glucose on erythrocyte membrane proteins. We demonstrated the protective role of glucose upon *in vitro* incubation of erythrocytes in solutions containing various concentrations of t-butyl peroxide glucose [32]. Blood cell counts in both CRF patient groups were not significantly different, but erythrocyte parameters were of note. Mean hemoglobin content and cell concentration were significantly higher in patients dialyzed with glucose-containing fluids compared to the group dialyzed with glucose-free fluid. At the same time, the HD-g(–) group was characterized by a significantly higher RDW value, suggesting better renewal of cells, in this case due to higher cell wear.

In summary, one may conclude that the presence of glucose in the dialytic fluid increases the erythrocytic activity of G6P-DH, which may be associated with a mechanism of defense against the consequences of ischemia in CRF patients. The presence of glucose increases the activity of the pentose phosphate pathway, which presumably enhances antioxidative defense. Oxidative stress accompanying hemodialysis in chronic renal failure patients damages mainly integral membrane proteins and erythrocyte cytoskeletal proteins, markedly affecting the survival of blood cells; however, a single hemodialytic procedure does not significantly change the protein profile of erythrocyte membranes.

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