Aggregation and elongation indices of erythrocytes of patients with chronic renal insufficiency relate to antioxidant capacity FRAP and CAT/SOD Ratio values

Agata PIETRZYCKA[1,*]

Karolina SŁOCZYŃSKA[2]

Aneta TELEGŁÓW[3]

Anna MARCHEWKA[3]

Maciej DROŻDŻ[4]

Marek STĘPNIEWSKI[1]

Henryk MARONA[2]

[1]Radioligand Laboratory of Pharmacobiology Department, Jagiellonian University, Medical College, Faculty of Pharmacy, 9 Medyczna Street, 60-688 Krakow, Poland

[2]Department of Technology and Biotechnology of Drugs, Jagiellonian University, Medical College, Faculty of Pharmacy, 9 Medyczna Street, 60-688 Krakow, Poland

[3]University School of Physical Education, Department of Clinical Rehabilitation, Al. Jana Pawła II 88, 31-571 Krakow, Poland

[4]Department of Nephrology, Jagiellonian University, Collegium Medicum, 15c Kopernika Str., 31-501 Krakow, Poland

The Head of the Pharmacobiology Department of Jagiellonian University, Medical College, Faculty of Pharmacy, 9 Medyczna Street, 60-688 Krakow, Poland: prof dr hab. Gabriel Nowak

The aim of the study was to evaluate multivariate correlation between selected rheological parameters and antioxidant defense biomarkers of red blood cells in patients with renal insufficiency undergoing hemodialysis. Measurement of hemorheological parameters was performed with Laser-Assisted Optical Rotational Cell Analyzer (LORCA). Superoxide dismutase (SOD) and catalase (CAT) activities were analyzed by Fridovich and Aebi methods respectively. Total antioxidant capacity, expressed as FRAP (ferric reducing ability of plasma) value was ascertained by the method of Benzi. 17 hemodialysis patients and 13 healthy control subjects were studied. It was observed that patients with renal insufficiency had significantly increased AI (aggregation index) when compared to healthy volunteers. Furthermore, these patients had significantly reduced index of deformability (elongation index - EI) in shear stress 0.58 Pa. It was found that dialyzed patients had markedly increased FRAP, which was accompanied by slight lowering of CAT/SOD ratio. Multivariate cluster analysis revealed that FRAP values were closely linked with AI whereas CAT/SOD ratio correlated with EI at the lowest shear stress pressures. On the basis of results, it could be supposed that imbalance between activities of CAT and SOD, may play crucial role in the impairment of red blood cells haemorheology in dialyzed patients.

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Zależność agregacji i wskaźników wydłużenia erytrocytów pacjentów z przewlekłą niewydolnością nerek od poziomu całkowitej zdolności antyoksydacyjnej FRAP i równowagi enzymów antyoksydacyjnych CAT/SOD Ratio

Celem pracy była wieloczynnikowa analiza zależności pomiędzy wybranymi wskaźnikami reologicznymi krwinek czerwonych a wskaźnikami obrony antyoksydacyjnej w grupie pacjentów z przewlekłą niewydolnością nerek, poddawanych dializoterapii. Pomiar wskaźników hemoreologicznych został wykonany na ektacytometrze (Laser-Assisted Optical Rotational Cell Analyzer -LORCA). Równowagę pomiędzy enzymami antyoksydacyjnymi dysmutazą ponatlenkową (SOD) i katalazą (CAT) wyrażono jako CAT/SOD Ratio. Wyniki: u pacjentów z przewlekłą niewydolnością nerek zaobserwowano znamiennie wyższy wskaźnik agregacji krwinek czerwonych oraz zmniejszoną zdolność erytrocytów do odkształcania w porównaniu do zdrowych ochotników. Ponadto, w grupie pacjentów dializowanych stwierdzono wyższy poziom FRAP i obniżoną wartość CAT/SOD Ratio. Wyniki wieloczynnikowej analizy skupień pokazują, że wartości FRAP mogą wpływać na wskaźnik agregacji AI, natomiast zaburzenia równowagi miedzy enzymami antyoksydacyjnymi mogą zmieniać odkształcalność krwinek czerwonych przy najniższych wartościach naprężeń

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Key words:
- haemodialysis
- erythrocyte
- elongation index
- aggregation
- superoxide dismutase
- catalase

Słowa kluczowe:
- hemodializa
- erytrocyty
- wskaźnik wydłużania
- agregacja
- dysmutaza ponadtlenkowa
- katalaza

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Adres do korespondencji:
Dr Agata Pietrzycka
Zakład Radioligandów
Katedry Farmakobiologii Wydziału Farmaceutycznego,
CM UJ , ul. Medyczna 9, 30-688 Kraków;
Tel. 12-6205-760; fax. 12-6205-400
e-mail: apietrzy1@cm-uj.krakow.pl

1. Introduction

Haemodialysis used in the treatment of the end stage of renal failure is often associated with change in shape [2,23,35] and increased incidence of the red blood cells (RBCs) deformability and higher erythrocyte aggregability [7,8,9,26,37]. It was noticed that the deformability of RBCs may be correlated with release of proinflammatory cytokines and tumour necrosis factor-α. Furthermore, activation of neutrophils and platelets was observed [4,5,18]. As the result of increase in free radical generation and/or depletion of antioxidant enzymes activity oxidative stress occurred [3,4,10,13, 30,31,41].

RBCs antioxidant defence primarily consists of enzymatic and non enzymatic sys-

tems. Enzymatic system includes superoxide dismutase (SOD), which catalyzes the dismutation of superoxide to hydrogen peroxide and catalase (CAT), which together with glutathione peroxidase, converts hydrogen peroxides to water and oxygen [4,14,16,19,36].

RBCs protection against the reactive oxygen species (ROS), provided by a non enzymatic antioxidant system, includes: a plasma membrane redox system (PMRS) and small thiol-containing peptides, albumin, bilirubin as well as uric acid [34]. PMRS maintenance redox state of sulfhydryl residues in membrane proteins, neutralize oxidants outside the cells, recycle α-tocopherol, reduce lipid hydroperoxides, and maintenance the extracellular concentration of ascorbic acid [34,38,39]. Total antioxidant capacity of a biological fluid and tissue is a combined effect of individual scavenging of these antioxidants [13,15,42]. Superoxide anions, high ATP levels, activated pump and altered composition and arrangement of membrane phospholipids and oxidized membrane proteins in RBCs, contribute with cell deformability in lower shear stress (elongation index) and higher values of shear stress at dissagregation (threshold shear rate) [4,5,23,35].

The aim of the study was to evaluate multivariate correlation between rheological parameters of red blood cells and antioxidant defence in patients with renal insufficiency undergoing haemodialysis.

Material and Methods

Blood rheology and chosen antioxidant parameters were evaluated in 17 adult patients (10 men, 7 women) with diagnosed end-stage renal disease undergoing haemodialysis twice time: just before and after dialysis session.

All patients, aged 61.2 ± 15.9 yr, were routinely dialyzed for 4 h three, times weekly, in the Department of Nephrology, University Hospital, in Krakow, using low-flux polysulfone membranes. 13 healthy (3 men, 10 women), non-smoking volunteers, who were not on any kind of medication, with normal renal function, aged 50.6 ± 11.7 yr served as the control group.

In the case of dialyzed group blood was drawn from the arterial side of the arteriovenous fistula just before dialysis session and was collected in polystyrene vacuum tubes containing EDTA, similarly as the venous blood obtained from the anticubital veins of control subjects.

In the first step, samples were prepared for RBCs deformability measurement as follows: 25ml of blood was diluted 200 times with 0.14 mM polivinylopyrrolidone (PVP, W=360 000, Sigma) in phosphate-buffered saline (PBS, viscosity at 37°C up to 31 mPa x s). Aggregation measurement was performed on a sample of 1-2 ml blood. Complete blood was oxygenated for 10-15 minutes before the measurement through the slow rotation in glass vessel.

For measurements of antioxidant parameters whole blood was centrifugated (2400 x g for 15 min) at the room temperature. The plasma was collected and retained for determination of the FRAP. Supernatant of red blood cells was washed 4 times with 150 mMol/l solution of NaCl, and lysed

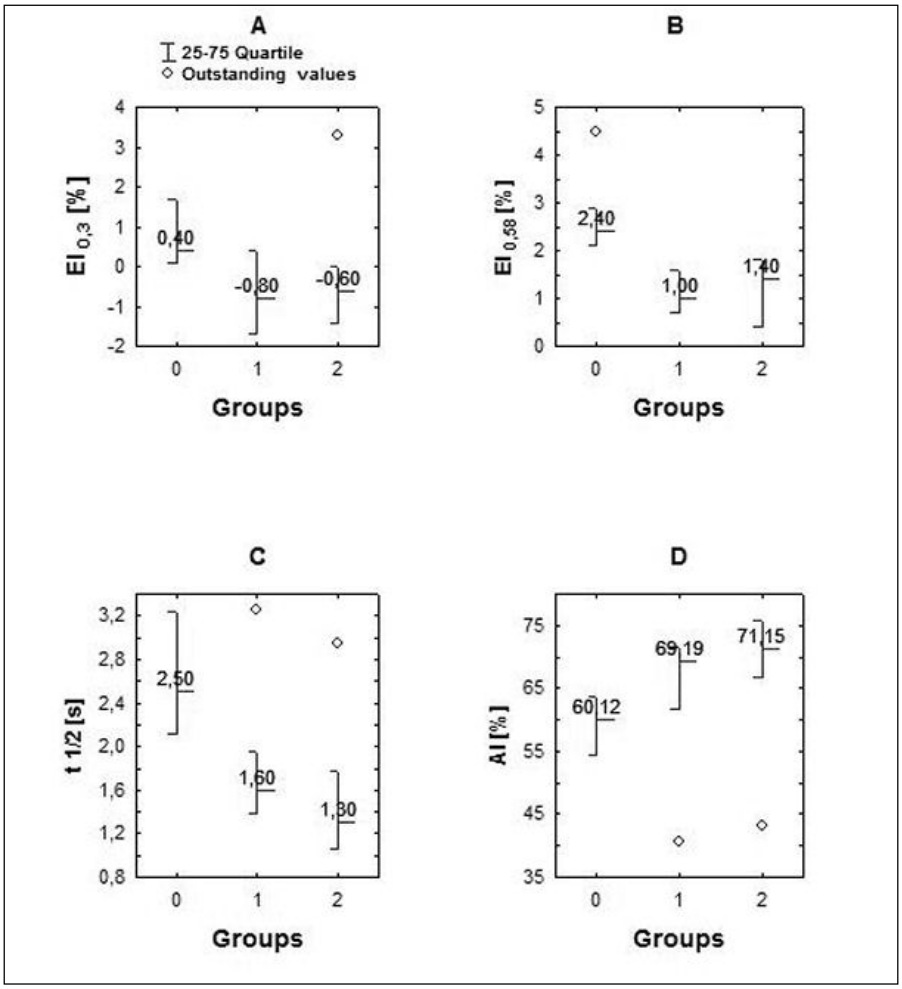

Figure 1

Comparison of elongation indices (EI) of red blood cells and aggregation parameters between healthy volunteers (0 Group) and patients with end stage renal insufficiency before (1 Group) as well after dialysis session (2 Group). Fig. A presents EI at 0.3 Pa shear stress; fig. B - EI at 0.58 Pa shear stress; fig. C - aggregation half time (t 1); fig. D - aggregation index (AI).

Porównanie wskaźników elongacji oraz wskaźników agregacji krwinek czerwonych między grupą zdrowych ochotników (Grupa 0) a grupą pacjentów z schyłkową niewydolnością nerek przed (Grupa 1) i po dializie (Grupa 2). Rycina. A - wskaźnik elongacji erytrocytów przy naprężeniu 0.3 Pa (EI0.3 [%]); Rycina. B - wskaźnik elongacji erytrocytów przy naprężeniu 0.58 Pa (EI0.58 [%]); Rycina. C - czas półtrwania agregatów krwinek czerwonych (t 1); Rycina. D - wskaźnik agregacji krwinek czerwonych (AI).

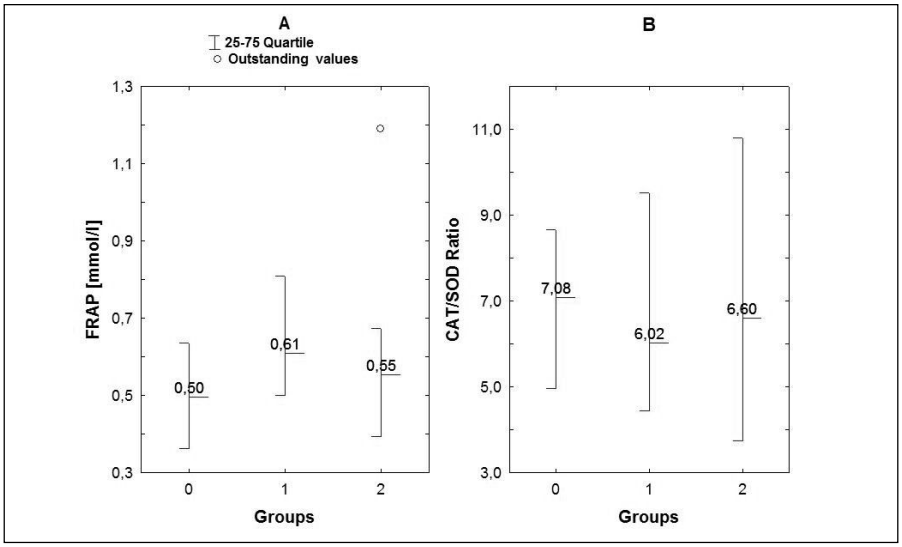

Figure 2

Comparison of total antioxidant capacity (Ferric Reducing Ability of Plasma - FRAP) and balance of catalase and superoxide dismutase, expressed as CAT/SOD Ratio, between healthy volunteers (0 Group) and patients with end stage renal insufficiency before (1 Group) as well after dialysis session (2 Group).

Porównanie całkowitej zdolności antyoksydacyjnej (Ferric Reducing Ability of Plasma - FRAP) oraz równowagi między katalazą a dysmutazą ponadtlenkową, wyrażoną jako CAT/SOD Ratio, między grupą zdrowych ochotników (Grupa 0) a grupą pacjentów z schyłkową niewydolnością nerek przed (Grupa 1) i po dializie (Grupa 2).

in 4.0 ml of ice-cold double distillated water. In hemolisate samples catalase (CAT) as well as superoxide dismutase (SOD) activities were measured.

CAT/SOD ratio was calculated after measurement of superoxide dismutase activity according to Fridovich's method [29] and catalase activity according to Aebi's method [1]. The total antioxidant capacity, expressed as the ferric reducing ability of plasma (FRAP) was measred spectrophotometrically by the method described by Benzi [6]. FRAP was expressed in (mmol/l).

Hemorheological parameters were performed with Laser-Assisted Optical Rotational Cell Analyzer (LORCA, Mechatronics, Instruments, The Netherlands). Technical details of LORCA as well as the protocol for measurement of RBC deformability and aggregation have been described in detail previously [20-22]. The device is capable of measuring two important hemorheological properties such as: red blood cells deformability [elongation index of RBCs in 0.3 Pa shear stress (EI in 0.3 Pa [%]); elongation index of RBCs in 0.58 Pa shear stress (EI in 0.58 Pa [%]); and aggregation parameters [the amplitude of aggregation (AMP); aggregation index (AI); aggregation half time (t 1); intensity of light scattering in the prior dissagregation (Isc disc); time when cells are round and not aggregated (Isc top)] and threshold shear rate (Threshold [1/s]).

All analyses were carried out using the statistical package "Statistics for Windows" (Statsoft, Poland, version 8.1). Test U of Mann-Whitney was used for analyzing differences between control and dialyzed subjects. In multivariate cluster analysis linkage rule called Ward's method was used.

Results

Figures 1 and 2 represent box plots of chosen antioxidant and rheological parameters in subjects participating in the study. The boxes include parameters' values from 25th to 75th percentiles. The central dash in boxes represent median values, and points outside the boxes show outstanding values.

Control group showed significantly higher median values of CAT/SOD Ratio and significantly higher elongation indices (EI) as well as aggregation half time (t 1) than dialyzed patients. Additionally significantly reduced FRAP level ($p \leq 0.05$) in healthy plasma when compared with hemodialyzed samples were observed. Aggregation index was increased significantly in dialyzed subjects as expected (figure 1 and 2; table I).

The main result of a cluster analysis is a tree diagram presented in the figure 3. FRAP values were closely linked with aggregation index (AI) and threshold shear rate (Threshold). CAT/SOD Ratio correlated with elongation index in 0.3 Pa shear stress, with elongation index in 0.58 Pa shear stress and aggregation half time (t 1). Other rheological parameters (Amp, Upstroke, Isc top, Isc disc) seemed to be not linked directly with antioxidant parameters, so that these data were excluded from further analysis.

Analysis of the % change of rheological and antioxidant parameters after dialysis session revealed the greatest change in red blood cells deformability as well as aggre-

Table I

Tests U of Mann-Whitney for significant differences between samples of healthy volunteers and samples of dialyzed patients before dialysis session.

Znamienne różnice w teście U Manna-Whitney'a między grupą zdrowych ochotników a grupą pacjentów z schyłkową niewydolnością nerek, poddanych dializoterapii.

	Median Control	Median Dialyzed pts	Z	Level p
Age [years]	55,00	61,00	-1,93	0,050
FRAP [mmol/l]	0,496	0,609	-1,80	0,047
$EI_{0,3}$ [%]	0,40	-0,80	2,49	0,010
$EI_{0,58}$ [%]	2,40	1,00	3,29	0,000
t 1/2 [s]	2,50	1,60	3,01	0,002
AI [%]	60,12	69,19	-2,93	0,002

Table II

% Change of the rheological and antioxidant parameters after dialysis session.

Procentowa zmiana wybranych wskaźników reologicznych oraz wskaźników obrony antyoksydacyjnej w grupie pacjentów z schyłkową niewydolnością nerek, po dializie.

	Median	25th Percentile	75th Percentile	Variation
Δ FRAP [%]	-9,38	-35,69	3,92	2689,72
Δ CAT/SOD Ratio [%]	9,64	-35,69	10,92	2689,72
Δ EI 0,30 [%]	25,00	-25,00	96,92	1696,42
Δ EI 0,58 [%]	40,00	-28,57	30,77	7793,10
Δ t 1/2 [%]	-18,75	-27,78	0,68	294,04
Δ AI [%]	2,84	0,48	8,43	24,33

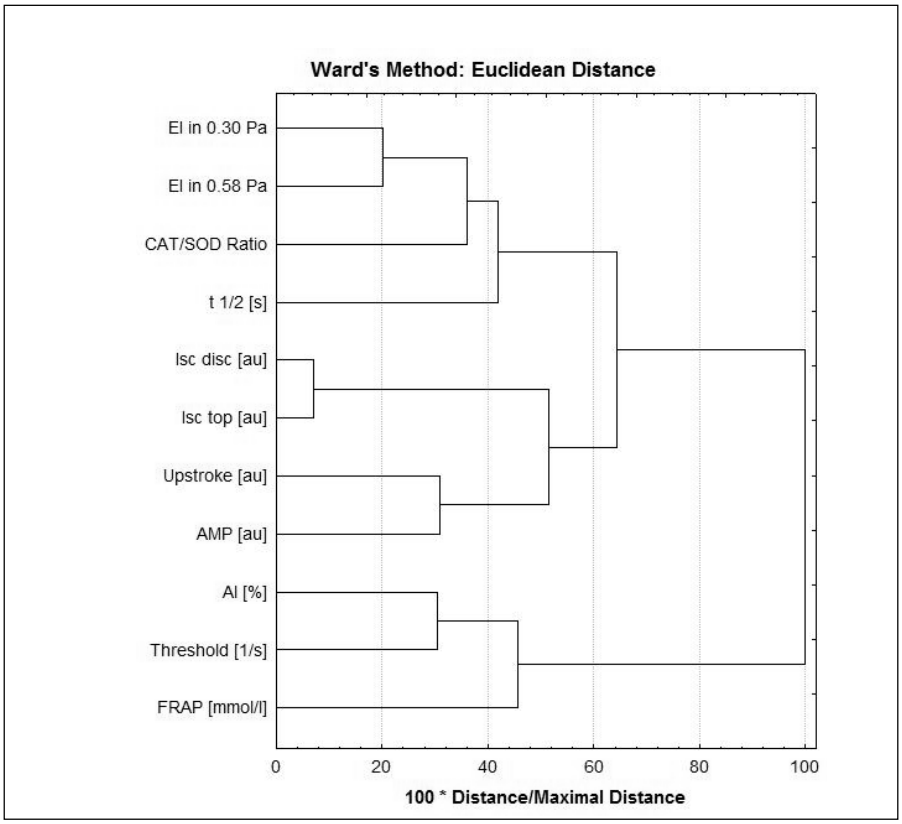

Figure 3

Multivariate cluster analysis of erythrocyte rheological properties and antioxidant parameters of patients with renal insufficiency.

Wieloczynnikowa analiza skupień właściwości reologicznych krwinek czerwonych oraz obrony antyoksydacyjnej w grupie pacjentów z schyłkową niewydolnością nerek, przed dializoterapią.

Notes: The horizontal axis represents the ratio of Euclidean distance to maximal Euclidean distance of the variable in the original data set. The links between variables are represented as U-shaped lines. The height of the "U" indicates the distance between the objects. Shorter distance between two variables suggests stronger coincidence.

Oś X przedstawia stosunek odległości Euklidesowej do maksymalnej odległości Euklidesowej, jaką zajmuje dana zmienna w przestrzeni. Linie w kształcie litery "U" przedstawiają związek (korelacje) między dwoma zmiennymi. Wysokość litery U wskazuje na odległość między dwoma zmiennymi w przestrzeni. Im ta odległość jest mniejsza, tym silniejsze oddziaływanie zachodzi między zmiennymi.

gation half time whereas aggregation of erythrocytes did not change (figure 1; table II).

Multivariate cluster analysis including % change of erythrocyte rheological properties and antioxidant parameters before dialysis session visualized a probable and close relationship between values of FRAP and CAT/SOD ratio before dialysis and change in aggregation index. Additionally, indirect link between alteration in EI in the low pressure and initial antioxidant markers was noticed (figure 4).

It was noticed that aggregation index rose proportionally with increasing values of CAT/SOD ratio. In the higher values of CAT/SOD ratio, occurring together with low FRAP, the largest aggregation of red blood cells was observed (figure 5).

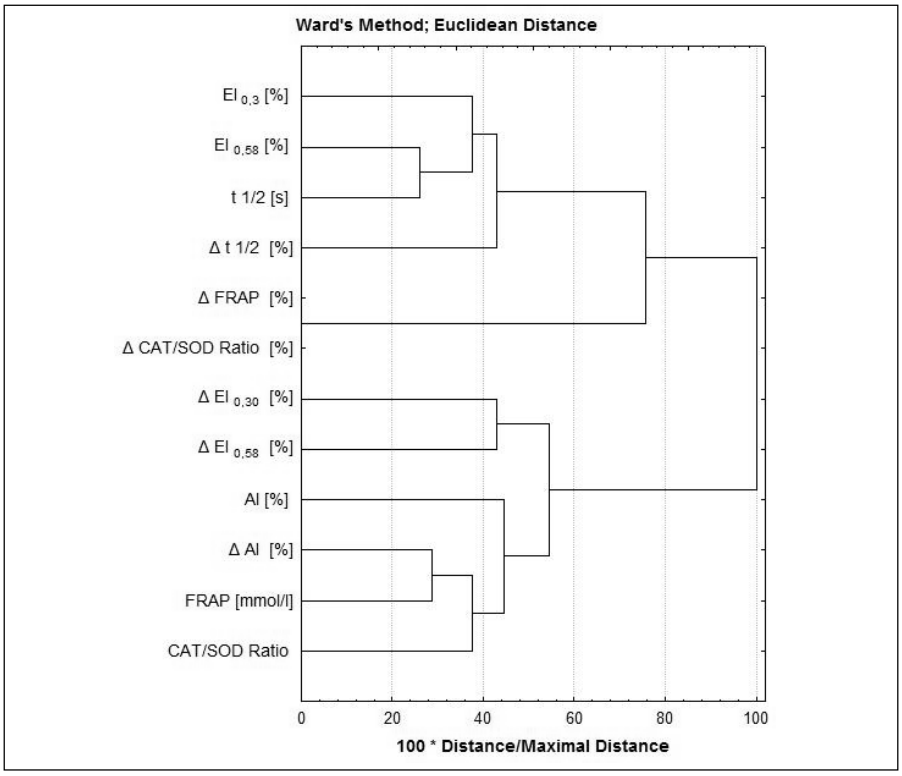

Figure 4

Multivariate cluster analysis of the % change of erythrocyte rheological properties and antioxidant parameters after dialysis session.

Wieloczynnikowa analiza skupień uwzględniająca % zmianę właściwości reologicznych krwinek czerwonych oraz obrony antyoksydacyjnej w grupie pacjentów z schyłkową niewydolnością nerek, po dializoterapii.

Notes: The horizontal axis represents the ratio of Euclidean distance to maximal Euclidean distance of the variable in the original data set. The links between variables are represented as U-shaped lines. The height of the "U" indicates the distance between the objects. Shorter distance between two variables suggests stronger coincidence.

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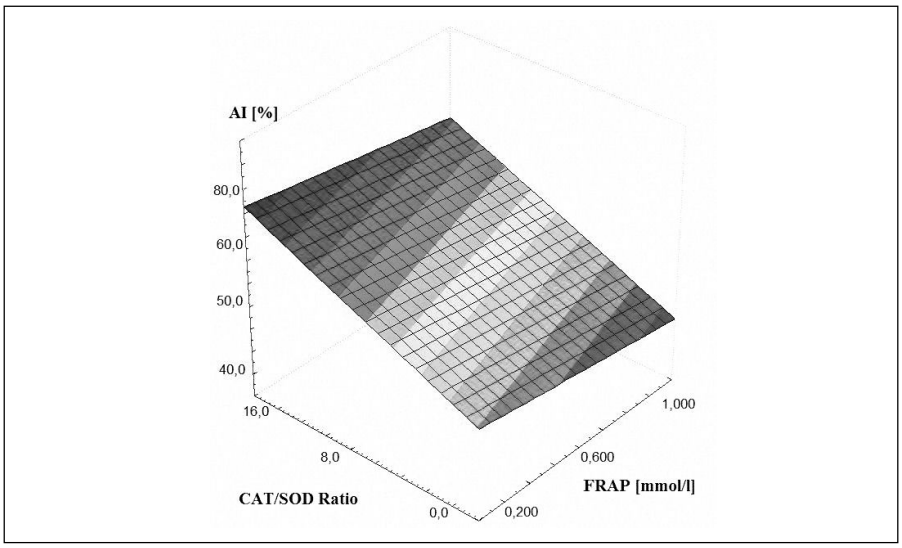

Figure 5

Linkage between Aggregation Index (AI [%]) FRAP and CAT/SOD Ratio of blood samples from dialyzed patients, before dialysis session.

Wykres powierzchniowy przedstawiający zależność między wskaźnikiem agregacji, całkowitą zdolnością antyoksydacyjną FRAP oraz równowagą między CAT oraz SOD wyrażoną jako CAT/SOD Ratio u pacjentów z schyłkową niewydolnością nerek.

Discussion

Complications of chronic renal failure predispose to the derangement and functions of erythrocyte membrane [5,8,33,36]. As a consequence rigidity of RBC increase and their survival reduce [37]. Results of the present study also demonstrate change in rheology of erythrocytes. It was observed that patients suffering from renal insufficiency had significantly increased aggregation index (AI) of red blood cells (RBCs) when comparing to healthy volunteers. Furthermore, these dialyzed patients characterized significantly lower deformability (figure 1; table I). Analysis of rheological aggregation and deformability of RBCs before and after dialysis session illustrated that elongation indices in shear stress of 0.3 and 0.58 Pa (EI 0.3 and EI 0.58) increased from 20 to 40 % of the initial values whereas AI was inconsiderably reduced (table II). These findings are analogical with reports of Nowak-Piszczek et. al. who found the improvement in deformability of cells in postdialysis samples [32,33].

Rheological alternations of red blood cells detected in patients undergoing hemodialysis may be due to structural and/or functional changes resulting from genetic factors and/or consequence of oxidative stress [5,8,9,25,26]. Multi factors such as activation of circulating leukocytes, generation of activated complement components due to contact of blood with bioincompatible membranes, transferring endotoxins from the dialyzate to the blood compartment and possibly other factors, lead to generation of highly reactive oxygen species (ROS). It is well known, that especially vulnerable to ROS are proteins of cells membranes. For example, oxidation of amino acid tyrosine, leads to the formation of dityrosine, protein aggregation, cross-linking, and fragmentation [12].

Recently, it was reported that advanced oxidation protein products (AOPP) are carried by oxidized plasma proteins, especially albumin and accumulate in subjects with renal disease. AOPP-albumin additionally, stimulate the oxidative burst and the synthesis of proinflammatory cytokines in neutrophils and monocytes. AOPPs, may directly impair HDL and lipid metabolism [28] and might therefore oxidize polyunsaturated fatty acids in membrane.

The insufficient antioxidant defence is reported as another reason of oxidative stress [8,13,16,19,38,40]. In the depletion of reactive oxygen species non-enzymatic components (total antioxidant capacity) and antioxidant enzymes play a role. Especially important seems balance between superoxide dismutase and catalase. For example, elevated SOD activity may imbalance cellular oxidant defence, resulting in enhanced oxidation due to the accelerated generation of hydrogen peroxide. This ef-

fect leads to activation of the second enzyme - catalase [37].

It was unexpectedly found that dialyzed patients had markedly increased total antioxidant capacity expressed, as ferric reducing ability of plasma (FRAP). Increase in FRAP was accompanied by slight lowering of CAT/SOD ratio (figure 2), however higher values of CAT/SOD ratio existed in almost 30% of haemodialyzed subjects (data available at authors). High values of CAT/SOD ratio may indicate large activity of catalase compensating accelerated dismutation of peroxide anions by SOD [24,25].

Estimating multivariate relations between variables, it was revealed that FRAP values were closely linked with AI whereas CAT/SOD ratio correlated with EI in 0.3 Pa shear stress, with EI in 0.58 Pa shear stress and t 1 (figure 3). Furthermore, it was also observed that change of aggregation index correlates both with initial values of FRAP and CAT/SOD Ratio (figure 4).

It worth to point out that aggregation index rose proportionally with increasing values of CAT/SOD Ratio (figure 5). In the higher values of CAT/SOD ratio, occurring together with low FRAP, the largest aggregation of red blood cells was observed.

Similar observation of the reduction in RBCs aggregation concomitant with significant decrease in SOD activities was reported by Kayatekin et. al., although authors observed no change in erythrocyte deformability [24].

Analogical, observation on positive correlation between deformability of red blood cells, total antioxidant status and superoxide dismutase, however in healthy humans, was reported by Chmiel et al. [11]. However, our study revealed for the first time complex relations between rheological and antioxidant parameters.

Increased activity of antioxidant enzyme (CAT) as a result of oxidative stress, is consistent with observations from another study conducted on type 2 non-insulin-dependent diabetes mellitus (NIDDM) [27]. Dursun et. al reported that activities of CAT was significantly higher in NIDDM hemolysate than in healthy blood donors.

On the contrary, activity of SOD in hemolisates of uraemic patients was reported to not differ from control (healthy) subjects [43].

On the basis of this fact and results of present study, it could be supposed that low level of total antioxidant capacity (FRAP) as well as imbalance between activities of catalase and superoxide dismutase, expressed as high values of CAT/SOD ratio, may play a key role in the impairment of red blood cells rheology in samples of dialyzed patients.

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