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Alterations of erythrocyte structure and cellular susceptibility in patients with chronic renal failure: Effect of haemodialysis and oxidative stress

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Abstract
The aim of this study was to investigate erythrocytes rheological behaviour, membrane dynamics and erythrocytes susceptibility to disintegration upon strong oxidative stress induced by dialysis or by external H_2O_2 among patients with CRF. EPR spectrometry was used to investigate alterations in physical state of cellular components. Generated ROS production induced: (1) significant increase of membrane fluidity in CRF erythrocytes treated with H_2O_2 ($p < 0.005$) and at 60 min of haemodialysis ($p < 0.05$), (2) significant decrease of cytoskeletal protein–protein interactions ($p < 0.005$) and (3) cellular osmotic fragility ($p < 0.0005$). H_2O_2 exacerbated these changes. Erythrocytes from CRF patients have changed rheological behaviour and present higher susceptibility to disintegration. Erythrocytes membrane characteristics indicate that CRF patients possess younger and more flexible cells, which are more susceptible to oxidative stress. This may contribute to the shortened survival of young erythrocytes in CRF patients.

Keywords: *Chronic renal failure, hydrogen peroxide, erythrocyte, rheology, cytoskeleton, osmotic fragility*

Abbreviations: *CRF, chronic renal failure; HD, haemodialysis; EPR, electron paramagnetic resonance; MSL, 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl; ISL, 4-iodoacetamide-2,2,6,6-tetramethylpiperidine-1-oxyl; 5-,12-,16-DS, 5-,12-,16-doxylstearic acid; RBC, red blood cell; AChE, acetylcholinesterase; ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; GSSG-R, glutathione reductase; GSH, glutathione; PBS, phosphate buffered saline; MDA, malondialdehyde; NaCl, sodium chloride; AGEs, advanced glycoxidation end-products; AOPP, advanced oxidation protein products; LMW-C, low molecular weight carbonyls; HNE, 4-hydroxy-nonenal; PUFA, polyunsaturated fatty acid.*

Introduction

It has been reported that free radicals play a crucial role in the pathophysiological pathways of different clinical and experimental renal diseases. Patients maintained on haemodialysis (HD), in particular those treated with cellulose membranes (e.g. cuprophan membranes), are chronically exposed to the oxidative stress as a result of predialitic-neutrophil activation through the action of complement-activated compounds [1]. Erythrocytes in chronic renal failure (CRF) patients are damaged mechanically by shear stress generated during flow through the dialyser and peristatic pumps and chemically by reactive oxygen species (ROS). Collision of erythrocytes and other

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blood cells as well as artificial materials leads to physical damage to the plasma membrane and shear induced erythrocyte haemolysis.

Reactive oxygen species such as hydrogen peroxide, hypochlorus acids and oxygen radicals: hydroxyl, superoxide and nitrogen oxide are released by phagocyting cells during the contact of blood with dialysis membranes [2,3]. The highest concentration of reactive oxygen species was detected at 20 min of haemodialysis [4,5]. Furthermore, free radicals production stimulated with phorbol ester increases during period of haemodialysis in the blood and inside the cells [6,7]. Physical and chemical plasma membrane damages lead to the release of haemoglobin, which in reaction with hydrogen peroxide can form ferryl or hydroxyl radicals, which are powerful oxidizing agents. However, the most prominent and effective representatives of oxidative stress in CRF patients are: isoprostanes, oxysteroles, 4-hydroxynonenal (HNE). For example, oxysteroles initiate atherosclerosis and increase the risk of coronary heart disease [8,9]. It was shown that both in serum and in cellular membrane not only polyunsaturated fatty acids (PUFA) are oxidized but also oxysterols [8,9]. However, creation of isoprostanes, which are detected in haemodialized patients, is related to inflammation [10,11].

Oxidative stress, which is associated with chronic renal failure, modifies molecules such as amino acids, lipids, carbohydrates and proteins [12]. The increased lipid peroxidation in plasma and erythrocytes as well as in plates and mononuclear cells has been reported in haemodialysed patients. Advanced glycoxidation end-products (AGEs), carbonyls (LMW-C) and advanced oxidation protein products (AOPP) are also examples of changes that occur in kidney failure as well as in haemodialysis treatment [12].

The above reported examples of biochemical alterations accompanying chronic renal failure may explain higher incidence of complications such as anaemia, atherosclerosis, accelerated ageing, cataract and dialysis-related amyloidosis [12–15].

ROS production in CRF patients can be additionally exacerbated by the depletion of antioxidative defenses [16,17]. Other causes of developed oxidative stress are advanced age, chronic inflammatory state, excessive parenteral iron administration and deficiency of vitamins C, E and selenium. In these conditions the main erythrocyte metabolic pathways are inefficient. For example, decreased level of NADPH and GSH and increased activity of glutathione reductase (GSSG-R) have been described [18].

Considering that erythrocyte biosynthesis capacity is very limited and that these cells have poor repair mechanisms, exposition to the oxidative stress may be mainly reflected in physical and/or molecular modifications. Oxidative stress can induce changes in composition and structure of erythrocytes components, which can affect their interactions, membrane structure and functions [19]. Moreover, lipid peroxidation causes depolarization of lipids bilayer and yields changes in the structural organization of membrane lipids that is reflected in membrane fluidity and, in consequence, can be important for physiological functions of erythrocyte. Those processes may contribute to the alterations in the enzymatic activity of membrane bound proteins and may result in protein–protein as well as protein–lipid interactions. Results obtained based on the EPR studies evidenced that during cell ageing *in vivo* rigidification of membrane lipids was observed, suggesting changes in lipid–protein interactions as an important factor in the decrease of lipid fluidity in aged cells [20]. Furthermore, acetylcholinesterase (AChE) activity also reflects changes in the chemical and physical properties of hydrophobic environment of the membrane and is changed with cellular age [21]. For example, in CRF patients higher activity of AChE was reported that is connected with intravenous erythropoietin (glycoprotein which stimulates erythrocytes production) [22]. Thus, it can be concluded that CRF patients had a higher number of young erythrocytes, which were less dense with higher membrane flexibility. Similar results are reported by other studies [23–25]. Besides, in CRF or dialysed patients many erythrocytes changes can occur after the cell enters the circulation [22]. Some of these changes are responsible for premature removal of the senescent cells [26].

Normal erythrocytes have a life-span of 120 days and reach this age contrary to uremic erythrocytes. Survival of the CRF patients cells is decreased as in patients treated with haemodialysis [7] because uremic erythrocytes are eliminated from the circulation earlier and a process of new cell production occurs to compensate for early loss of old cells. Hence, the uremic blood is enriched with younger cells. We suggest that increased susceptibility of erythrocytes to oxidative changes may be responsible for their shortened survival.

The aim of this study was to evaluate erythrocyte susceptibility to disintegration and to estimate the changes in erythrocyte membrane dynamics before, during and after haemodialysis. Furthermore, the influence of oxidative stress induced by hydrogen peroxide (H_2O_2) on physico-biochemical properties of erythrocytes membrane and cellular susceptibility has been presented among patients with CRF compared to the healthy subjects. As markers of erythrocytes alterations, the conformational changes of membrane proteins (mainly in the complex of spectrin-actin) and membrane dynamics described by membrane fluidity were estimated. The latter one is an important factor of cellular rheological behaviour and membrane microviscosity. Additionally, we

investigated erythrocytes internal viscosity and osmotic fragility.

Materials and methods

Chemicals

4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MSL), 4-iodoacetamide-2,2,6,6-tetramethylpiperidine-1-oxyl (ISL) 5-,12-,16-doxylstearic acids (5-DS, 12-DS and 16-DS) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were analytical grade products from POCh (Gliwice, Poland).

Subjects

The study population consisted of eight patients with mild-to-advanced chronic renal failure (CRF), who were treated at the Department of Internal Medicine at the Medical University in Lodz. Among them six patients had glomerulonephritis, one had diabetic nephropathy and one had polycystic kidney disease. Patients were dialysed with the use of cuprophan dialysers DIACAP CE 1600. The mean age of recruited patients was 58 ($\pm$11) years. All patients received erythropoietin. The control group of healthy subjects was recruited among volunteers of the Outpatient Center of Medical University in Lodz. The mean age of the control group was 46 ($\pm$15) years.

Erythrocytes and erythrocyte ghosts

Venous blood samples were collected (in standard sterile polysteryne vacuum tubes with heparine 16 U/ml of blood) before dialysis, at 20 and 60 min of dialysis session and 4 h after dialysis. Packed cells were suspended in phosphate buffered saline (PBS) to a haematocrit of 50%. Isolated erythrocytes from healthy donors as well as from patients with CRF before haemodialysis were treated with 100 μM H_2O_2 at room temperature for 1 h and then washed with PBS, pH 7.4.

Erythrocyte ghosts were prepared by the method of Dodge et al. [27] with the modification including hypertonic lysis using 20 mM sodium phosphate buffer (pH 7.4) at 4°C. The ghosts were successively washed with 20, 10 and 5 mM phosphate buffer (pH 7.4) and finally suspended in 5 mM phosphate buffer (pH 7.4) at 4°C.

Spin labelling of erythrocytes and erythrocyte ghosts

Erythrocytes were labelled with 5-,12-,16-doxylstearic acid (5-DS, 12-DS and 16-DS) by introduction of the doxyl derivatives in ethanol into the erythrocyte suspension and incubated for 30 min at room temperature. The final ethanol concentration in the erythrocyte suspension was less than 0.05% (v/v). From the EPR spectra the ratio h_{+1}/h_0 was calculated, with h_{+1} representing the height of the low-field line and h_0 representing the height of the middle-field line of the spectrum. This ratio is correlated with lipid bilayer fluidity and serves as a semi-quantitative measure of acyl side chain flexibility [28].

To investigate conformational changes of membrane proteins, 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MSL) and 4-iodoacetamide-2,2,6,6-tetramethylpiperidine-1-oxyl (ISL) were used, which bind covalently to the $-$SH groups of membrane proteins. Erythrocyte membranes were labelled with MSL or ISL using 2 μl of ethanol solution with 100 mM MSL or ISL per 1 ml of membrane suspension ($\sim$3 mg/ml of protein), and incubated for 1 h at 4°C. Unbound spin label was removed through several washings with cold phosphate buffer. The effect of induced oxidative stress on the ratio of weakly-to-strongly immobilized residues of erythrocyte membrane-bound MSL spin labels (h_w/h_s) was studied [29].

In the case of ISL, the mobility of attached spin label was estimated by calculating the rotational correlation time (τ_c) according to the Kivelson [30] formula.

Electron Paramagnetic Resonance (EPR) spectra were obtained at room temperature (22°C) using a Bruker ESP 300 E spectrometer, operating at a microwave frequency of 9.73 GHz. The instrumental settings were as follows: centre field 3480G; scan range 80G; modulation frequency 100 kHz; modulation amplitude 1G.

Osmotic fragility

Osmotic fragility was estimated according to the spectrophotometric method described by Marimoto et al. [31]. Changes in osmotic fragility were presented as a concentration of sodium chloride (NaCl), for which 50% of erythrocytes in the investigated groups underwent haemolysis (C (50%)).

Erythrocyte internal viscosity

Erythrocyte internal viscosity was monitored as described by Morse [32] using tempamine, which easily goes through the membrane and stays unbound inside of erythrocyte. Tempamine solution: 1 μl of 0.1 mol/l was added to the isolated erythrocytes and next erythrocytes were washed with 5 mmol/l buffer, pH = 7.4, containing 80 mmol/l potassium ferricyanide. Potassium ferricyanide as a broadening agent was used to eliminate the signal derived from an excess amount of extracellular spin label. From EPR spectra the erythrocytes internal viscosity was calculated according to the following formula:

$$\eta = \frac{\tau_c(RBC)}{\tau_c(H_2O)}\eta_{H_2O} \quad (1)$$

where τ_c(RBC) is rotational correlation time inside the erythrocyte, $\tau_c(H_2O)$ is rotational correlation time in water and η_{H2O} is water viscosity equal to 1cP.

Statistical analysis

Normality of distributions was tested using the Shapiro-Wilk test. The significance of the difference between couples of means (control and investigated probes as well as the differences among the probes) was assessed by the Tukey test at $\alpha < 0.05$ significance level.

Results

The lipid membrane fluidity was estimated at different depths of lipid bilayer using three spin labelled fatty acids: 5-, 12- and 16-doxylsteric acid.

Table I presents the results, which indicate significant differences in lipid fluidity between erythrocytes from CRF patients treated with H_2O_2 and healthy subjects on the depth of the 5th and 12th carbon atom of the phospholipids chain ($p < 0.005$). On the other hand there were no statistically significant changes in the deeper region on the 16th carbon atom of the phospholipids acyl chain. A significant decrease in lipid fluidity was detected on the depth of the 12th carbon atom of the phospholipids chain in erythrocyte from CRF patients ($p < 0.05$). Table II shows results from the examination of the effect of haemodialysis on erythrocytes membrane fluidity that indicate significant differences between control and patients with CRF at 60 min of haemodialysis on the depth of the 12th and 16th carbon atoms of the phospholipids chain ($p < 0.05$). In this case, no significant changes were detected in the polar region of membrane bilayer but a tendency of increasing fluidity during haemodialysis was observed.

We detected a remarkable increasing tendency in lipid fluidity on the different depth of membrane bilayer as an effect of haemodialysis at the different time of this process in comparison with the control group. However, 4 h after the dialysis session a decreasing tendency in lipid fluidity was observed. The values of h_{+1}/h_0 parameter were comparable with values obtained at 20 min of haemodialysis.

Physical state of membrane proteins was estimated using maleimide and iodoacetamide spin labels, which form covalent bounds with –SH groups [33]. An increase of $\sim 35\%$ of the h_w/h_s ratio was observed in ghosts isolated from CRF erythrocytes in comparison with control. Treatment of both groups of erythrocytes with H_2O_2 led to an increase in the h_w/h_s ratio. Stronger changes were observed in control erythrocytes (40%) than in erythrocytes from CRF patients (19%).

Table III presents the effect of H_2O_2 on the conformational changes of cytoskeletal proteins. The analysis of maleimide spectrum and calculation of the h_w/h_s ratio show a statistically significant increase of this parameter in erythrocytes from CRF patients and control healthy subjects as well as the difference between erythrocytes from CRF patients treated with H_2O_2 and control healthy subjects treated with H_2O_2 ($p < 0.005$). The increase in this parameter indicates a decrease in cytoskeletal protein–protein interactions and an increase in segmental motion of spin-labelled proteins. This confirms results about the changes, which were found for the calculated parameter of rotational correlation time. Values for τ_c tend to be negatively correlated with the ratio h_w/h_s. A decrease ($\sim 19\%$) in the relative correlation time (increase in the motion of spin label residue) of ISL in the ghosts from CRF erythrocytes in comparison with control was observed. However, we found a significant decrease ($\sim 14\%$) of correlation time in the group of erythrocytes from healthy subjects upon H_2O_2 treatment ($p < 0.05$). Thus, the decrease in τ_c was observed in erythrocytes from CRF patients and in erythrocytes from CRF patients treated with H_2O_2 when compared to healthy control subjects ($p < 0.005$). These changes indicate an increase in the segmental motion of labelled proteins in patients with CRF and similarly in erythrocytes from CRF patients treated with H_2O_2 (Table IV).

Changes in erythrocytes membrane dynamics and organization caused cellular haemolysis estimated by osmotic fragility assay. They are shown in Table V. The results suggest that induced ROS production

Table I. Effect of hydrogen peroxide on erythrocytes membrane fluidity from healthy subjects and patients with CRF before dialysis (mean±SD).

	Lipid fluidity on the different depth of erythrocyte membrane bilayer h_{+1}/h_0			
Spin labels	Control	Control+H_2O_2	CRF	CRF+H_2O_2
5-DS	0.271±0.023, $n=26$	0.279±0.015, $n=8$	0.282±0.017, $n=24$	0.395±0.062***, $n=9$
12-DS	0.426±0.034, $n=26$	0.448±0.011, $n=8$	0.416±0.013*, $n=24$	0.511±0.028***, $n=9$
16-DS	0.676±0.021, $n=23$	0.686±0.039, $n=8$	0.679±0.021, $n=25$	0.661±0.009, $n=9$

***indicates significant difference between erythrocytes from healthy subjects and CRF patients treated with H_2O_2 at $p<0.005$.
*indicates significant difference between erythrocytes from healthy subjects and CRF patients at $p<0.05$.

Table II. Effect of haemodialysis on erythrocytes membrane fluidity in patients with CRF (mean ± SD).

	Lipid fluidity on the different depth of erythrocyte membrane bilayer h_{+1}/h_0			
Spin labels	Control	20 min HD	60 min HD	4 h after HD
5-DS	0.271 ± 0.023, $n = 26$	0.284 ± 0.019, $n = 9$	0.283 ± 0.012, $n = 8$	0.289 ± 0.013, $n = 8$
12-DS	0.426 ± 0.034, $n = 26$	0.431 ± 0.038, $n = 14$	0.46 ± 0.029*, $n = 11$	0.433 ± 0.044, $n = 9$
16-DS	0.676 ± 0.021, $n = 23$	0.685 ± 0.023, $n = 14$	0.701 ± 0.025*, $n = 14$	0.682 ± 0.033, $n = 8$

*indicates significant difference between healthy subjects and patients with CRF at 60 min of haemodialysis at $p < 0.05$.

during haemodialysis leads to the oxidative alterations in membrane components, which were significantly reflected in cellular lysis. The results indicate a significant increase in erythrocyte membrane osmotic susceptibility in CRF patients and in erythrocytes from CRF patients treated with H_2O_2 as well as healthy subjects treated with H_2O_2 ($p < 0.0005$).

We did not observe significant changes in erythrocyte internal viscosity. This suggests that induced oxidative stress did not cause detrimental changes in the internal components. However, a decreasing tendency was observed for control and urine erythrocytes treated with hydrogen peroxide.

Discussion

The aim of this study was to investigate erythrocyte susceptibility to disintegration and to estimate the changes in erythrocyte membrane composition as well as its rheological behaviour.

We investigated membrane fluidity at different times during haemodialysis as well as 4 h after this treatment. Analysis at 20 min of haemodialysis was motivated by a previously reported results about an increase in the production of superoxide anion and hydroxyl radical [34]. Moreover, after 20 min of ongoing dialysis a change in superoxide dismutase (SOD) activity and elevation of the malondialdehyde (MDA) level in the plasma of CRF patients has been reported [35]. However, 60 min of ongoing haemodialysis was chosen because at this time of treatment the highest amount of retained toxins is removed. Furthermore, the investigations of erythrocytes membrane were also performed 4 h after dialysis treatment. In HD patients oxidative stress appears to be a consequence of the accumulation of pro-oxidant compounds in the blood, depletion of antioxidants and activation of neutrophils or platelets relevant to blood–membrane interactions, which trigger ROS production. However, introduction of hydrogen peroxide was aimed at investigating erythrocyte membrane properties in CRF as well as in HD patients compared to healthy subjects. It has been dictated by the elevation of cellular membrane susceptibility to oxidized factors in both groups of patients.

Hydrogen peroxide was chosen because in the human body it can act as a cytotoxic agent, usually at the level ≥ 50 μM. Depending on its concentration, length of exposure and used cell type, it can lead to cellular damage, apoptosis or necrosis [36]. Hydrogen peroxide is a rather poorly reactive molecule to directly induce lipid peroxidation. However, its toxicity occurs through the Fenton reaction, with oxidation of transition metals and production of hydroxyl radical. In erythrocytes, the haemoglobin can be oxidized leading to the generation of its ferryl form, which is a strong oxidant that directly initiates lipid peroxidation and oxidative alterations in cellular membrane [37,38].

Our results reveal changes in erythrocyte membrane integrity at 60 min of haemodialysis and in CRF patients erythrocytes treated with hydrogen peroxide. Induced oxidative stress during dialysis increased lipid fluidity. Introduction of hydrogen peroxide to the erythrocytes of CRF patients also triggered similar results.

Lipid fluidity examination showed that erythrocytes from CRF patients present higher susceptibility to induced oxidative stress than erythrocytes collected from a healthy control group. Induced oxidative stress during haemodialysis or by introduction of hydrogen peroxide in erythrocytes from CRF patients led to lipid peroxidation causing depolarization of the lipids bilayer and triggering changes in the structural

Table III. Effect of hydrogen peroxide on conformational state of erythrocytes membrane proteins labelled with MSL from healthy subjects and patients with CRF (mean ± SD).

	Conformational state of erythrocyte membrane proteins h_w/h_s		
Control	Control + H_2O_2	CRF	CRF + H_2O_2
2.244 ± 0.292, $n = 8$	3.419 ± 0.319***, $n = 6$	3.285 ± 0.262***, $n = 7$	3.922 ± 0.322***, $n = 7$

***indicates significant difference between erythrocytes from CRF patients and control healthy subjects and between erythrocytes from CRF patients treated with H_2O_2 and control healthy subjects treated with H_2O_2 at $p < 0.005$.

Table IV. Effect of hydrogen peroxide on rotational correlation time for ISL bound to erythrocytes membrane proteins from healthy subjects and patients with CRF (mean ± SD).

Rotational correlation time (τ_c) (s)			
Control	Control + H_2O_2	CRF	CRF + H_2O_2
7.83 ± 0.88, $n = 6$	6.772 ± 0.549*, $n = 5$	6.36 ± 0.285***, $n = 7$	6.508 ± 0.606***, $n = 8$

***indicates significant difference between erythrocytes from CRF patients and control healthy subjects and between erythrocytes from CRF patients treated with H_2O_2 and control healthy subjects at $p < 0.005$.
*indicates significant difference between erythrocytes from healthy subjects treated with H_2O_2 and control at $p < 0.05$.

organization of membrane lipids that is reflected in membrane fluidity. Lucchi et al. [39,40] observed a significant increase of MDA production after incubation with the oxidative stress-inducing agent t-butyl hydroperoxide in patients dialysed with Cuprophan membrane. In our study on the level of the 12th carbon atom of the acyl chain in erythrocytes from CRF patients, a significant decrease in lipid fluidity was detected. It can be explained by the presence of double bonds in this region, which upon peroxidation lead to the formation of hydroperoxides or may create lipid–lipid and lipid–protein bindings. This may be the reason for membrane rigidity on the depth of the 12th carbon atom of the phospholipids chain. Detected presence of echinocytes in CRF patients [41] additionally confirms our results. However, introduced hydrogen peroxides induced lipid fluidity through the disintegration of peroxidized lipids. In erythrocyte from end-stage CRF patients the increase of MDA level was detected after incubation with t-butyl hydroperoxide, which means higher sensitivity to the oxidative stress [40].

In uremia, erythrocytes exhibit an increase of MDA, which suggests that the increase of lipid fluidity in CRF patients is correlated with lipid membrane peroxidation [12]. As a result of this process the fragmentation of lipid acid chain, loss of membrane material and change in the ratio of cholesterol and phospholipids additionally explains the reason for increasing lipid fluidity [22,42]. As a consequence, these processes further deteriorate physiological functions of erythrocyte and may be the reason of cellular lysis. Furthermore, these changes in lipids composition and physical properties of the membrane may result in erythrocyte ageing or premature removal from the circulation [22]. Changes in lipid membrane fluidity can result due to formation of conjugated hydroperoxide or fragmentation of unsaturated fatty acids chain in lipid molecules. The level of hydroperoxide was 3.2-fold higher in haemodialysed patients than in healthy subjects. The level of leukotriene was 1.7-fold above the control and the activity of 5-lipooxygenase was 2.4-fold higher than in control donors [43].

In order to extend our analysis of membrane dynamics we investigated the conformational state of membrane proteins. Through the analysis of the structure and composition of lipid–protein interface it is possible to provide full information about changes in membrane properties and organization.

Using a MSL spin label, we observed significant changes in conformational state of erythrocyte cytoskeletal proteins. In our study the influence of exogenous hydrogen peroxide on membrane proteins resulted in a significant increase of the ratio h_w/h_s, indicating an increase of spin label mobility, which was bound with membrane proteins after incubation with added oxidant. The h_w/h_s ratio generally increases during protein denaturation and degradation (e.g. by proteinases) [44,45], which is connected with the increase of segmental motion of spin-labelled proteins. Therefore, we interpret our results as the effect of the dissociation of spectrin–actin complex, in view of the fact that 75–90% of the total amount of attached spin labels is bound to this complex [29,46].

It has been shown that membrane proteins in erythrocytes are more sensitive to oxidative stress than lipids [47]. The ratio of h_w/h_s was $\sim$35% higher in the membranes isolated from CRF patients than in control. This result clearly shows profound changes in membrane cytoskeleton of erythrocytes in uremia.

Table V. Effect of hydrogen peroxide on osmotic fragility of erythrocytes from healthy subjects and patients with CRF (mean ± SD).

C (50%) (mmol/dm^3)			
Control	Control + H_2O_2	CRF	CRF + H_2O_2
71.25 ± 0.25, $n = 7$	76.58 ± 0.59****, $n = 12$	78.56 ± 0.38****, $n = 8$	87.52 ± 0.61****, $n = 8$

****indicates significant difference between erythrocytes from CRF patients and control healthy subjects and between erythrocytes from CRF patients treated with H_2O_2 and control healthy subjects treated with H_2O_2 at $p < 0.0005$.

It is worthwhile to mention that an increase in the h_w/h_s ratio was much higher in control erythrocytes (40%) than in erythrocytes from CRF patients (19%) upon H_2O_2 treatment, but CRF erythrocytes were damaged earlier in the blood of uremic patients. This may suggest that we observed a saturated state in membrane protein oxidation. The 'total oxidative stress' induced in uremia and hydrogen peroxide in CRF erythrocytes was 61% higher than in healthy controls.

Furthermore, conformational changes in membrane proteins confirmed results from membrane fluidity, indicating that erythrocyte membranes from CRF patients were more susceptible to oxidative changes induced by an external agent. The calculated parameter for CRF erythrocytes treated with H_2O_2 was 61% higher compared to healthy controls and only 19.4% higher compared to CRF patients. It becomes more understandable that erythrocytes of CRF patients present higher susceptibility to oxidative alterations and cellular membrane destabilization. These results confirm similar findings from erythrocytes treated with H_2O_2 in whole blood *in vitro* where increases in protein degradation have been reported [48].

The obtained results are supported by the values of rotational correlation time for iodacetamide spin label. Results show a significant decrease of rotational correlation time of iodocetamide spin label. It suggests that developed medical conditions had significant modulatory actions on the physical state of cytoskeletal proteins.

We explain the conformational changes of cytoskeletal proteins as the result of the oxidative process induced by hydroxyl radical generated in Fenton reaction after erythrocyte incubation with H_2O_2. Hydroxyl radical can initiate protein –SH groups peroxidation, that is associated with the promotion of both fragmentation and aggregation of membrane proteins and reflects conformational changes in the actin–spectrin complex [47]. The possible role of nitric oxide (NO) and reactive nitrogen species (RNS) in pathologic conditions of CRF patients (including uremia) has previously been suggested [49]. If NO is generated in these pathologic conditions through, for example, leucocyte activation, then peroxidation of erythrocyte membrane protein thiols can be conducted [50].

Our results show that generated strong oxidative stress by introduced external agent and direct contact of erythrocytes with dialysis membrane induces alterations in erythrocyte membrane composition as well as in its dynamics, which was reflected in cellular haemolysis. We found a significant rise in erythrocyte membrane osmotic fragility in erythrocytes from CRF patients. The observed decrease in membrane osmotic resistance was additionally deepened by added hydrogen peroxide. This observation helps explain the higher susceptibility of erythrocytes from CRF patients to oxidative stress. The measurement of osmotic fragility of erythrocytes is frequently used for diagnosis of haemolytic disease, alterations and destruction in plasma membrane as well as investigations of permeability through the membrane [51]. Higher osmotic fragility was observed after haemodialysis but it was suggested that parathyroid hormone is probably a major factor affecting erythrocytes osmotic fragility in CRF patients [52].

In conclusion, our experiment showed significant alterations in erythrocyte membrane composition and susceptibility caused by enhanced oxidative stress in CRF patients. Inducing oxidative stress by an external agent or exposing erythrocytes to chronic mechanical trauma during dialysis provoked deeper cellular membrane destabilization. The observed changes can contribute to the decrease of erythrocytes life span in CRF patients or other complications like anaemia, cardiovascular diseases or atherosclerosis. In light of our findings, the increased membrane fluidity and decreased cytoskeletal protein interactions characterize rheological behaviour of young cells and may explain the increase of the number of younger and more flexible erythrocytes (less dense cells) in CRF patients [20,22,24–26,53]. Strong lipid peroxidation was reflected by changes in membrane fluidity and led to the occurrence of cellular lysis as a consequence of the described processes. Therefore, membrane biochemical and biophysical alterations of the structure can be responsible for shortened survival of young erythrocytes in CRF patients.

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