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Investigation of albumin properties in patients with chronic renal failure

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Abstract
The aim of this study was the investigation of HSA properties and its structural changes after modification induced *in vivo* among patients with CRF who underwent haemodialysis. Application of different fluorescent dyes allowed the investigation of different regions of albumin molecule using ANS, bis-ANS, piren, piren maleimide and fluorescein isothiocyanate. As markers of oxidative modification, the total protein thiol, carbonyls, glycosylated plasma proteins and hydroperoxide were estimated in plasma. Additionally, this study investigated plasma viscosity and total antioxidant capacity (TAC) of the plasma. Results show that haemodialysis provoked significant changes in conformational properties of plasma albumin, which resulted in the loss of its biological functions. These findings suggest that oxidative stress and glycation of proteins in plasma are developed during haemodialysis. The results depict that one of the features of uraemia is the presence of signs of oxidative stress before haemodialysis. Nevertheless, oxidative stress and glycation of proteins in plasma are exacerbated during haemodialysis and are a complex process.

Keywords: *Albumin, chronic renal failure, haemodialysis, oxidative stress, glycosylation*

Abbreviations: *CRD, carbonyl reactive derivatives; RCD, reactive carbonyl derivatives; AOPP, advanced oxidation protein products; HAS, human serum albumin; CRF, chronic renal failure; HD, haemodialysis; EPR, electron paramagnetic resonance; ANS, ANS-1-anilino-8-naphthalenesulfonic acid; bis-ANS, 4,4′-Bis(1-anilino-8-naphtalenesulfonic acid); dipotassium salt; FITC, fluorescein isothiocyanate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); RBC, red blood cell; ROS, reactive oxygen species; TAC, total antioxidant capacity; SOD, superoxide dismutase; PBS, phosphate buffered saline; MDA, malondialdehyde; NaCl, sodium chloride*

Introduction

Free radicals play a key role in 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 predialytic-neutrophil activation through the action of complement-activated compounds [1]. Reactive oxygen species (ROS) 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 oxygen free radicals was detected at 20 min of haemodialysis [4,5] and was correlated with neutropenia occurring during haemodialysis at the same time [6]. Activation and degranulation of neutophils trigger oxidative burst, which causes oxidative damages of biological structures of proteins, lipids or DNA. Moreover, due to the fact that interleukins and anaphylatoxins produced during HD sessions are

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potent activators for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme that is responsible for over-production of reactive oxygen species (ROS), this may constitute a link between leukocyte activation and cell or organ toxicity [7].

Biochemical and structural modifications of proteins induced by oxidative attack may lead to functional alterations and in particular to a progressive loss of their metabolic, enzymatic or immunologic properties. Proteins are among some of the main targets of oxidation in plasma [8] and their thiol groups are very susceptible to reactions with ROS. Owing to that, modified plasma proteins can be considered as an important biomarker of oxidative stress *in vivo* due to its relatively long half-lives and well-defined cellular pathway.

It has been shown that chronic renal failure is characterized by the decreased antioxidant defences, decreased antioxidant enzyme activities and loss of low molecular weight antioxidants [9]. Since extracellular fluids contain small amounts of antioxidant enzymes, albumin has been proposed as a major antioxidant [10], which protects erythrocytes, other cells and lipoproteins against peroxidation. In the plasma of dialysed patients significant increase of advanced oxidation proteins products (AOPP), such as oxidized albumin, has been reported [10]. Besides, the protein molecules may also be attacked by carbonyl compounds, which can be introduced into proteins by modification of amino acids.

The plasma levels of urea in CRF patients are greatly increased (30–50 mM), while in normal physiological conditions they are 3–7 mM. Higher level of urea leads to carbamylation of aminoacids, peptides and proteins. Carbamylation is a result of permanent exposure and reaction with urea derived cyanate. The active form that reacts with $-NH_2$ groups of proteins is isocyanic acid, which is in equilibrium with cyanate. It has been shown that carbamylation alters protein conformation, decreases or inhibits enzymes activities, changes charge of proteins [11,12] and depletion of ascorbate in organism [13]. Thus, the appearance of carbonyl groups is a presumptive evidence of oxidative modifications in proteins.

Human serum albumin (HSA) exposed to oxidative stress alters its conformational and functional properties resulting in the changes of biological functions. HSA possesses 17 disulphide bridges and one free thiol group, which are oxidized by reactive oxygen species. ROS can also lead to the modifications of aminoacids residues and then to the albumin aggregation or fragmentation. Albumin can be also oxidized with hypochlorous acid and hydrogen peroxide. Moreover, heparin binding to albumin induces oxidative reactions, which are responsible for the increase in the carbonyl content of the protein together with its higher susceptibility to tryptic digestion. However, only a small percentage of albumin molecules underwent fragmentation in the presence of heparin with glucose [14].

Recently, phagocytes have been proposed as the most important source of ROS. In the presence of myeloperoxidase (MPO), which catalyses the reaction between chloride ion and hydrogen, peroxide neutrophils produce a large amount of hypochlorous acid (HOCl), a very toxic and oxidizing agent. The oxidation induced by mild oxidants (H_2O_2 or hydroperoxide radical) leads to the creation of –S-S-bridges, however strong oxidants, e.g. HOCl, trigger SOx (SOH, SO_2H, SO_3H) formation. Investigations showed that HSA treated with HOCl *in vitro* and *in vivo* generated AOPP, which caused oxidative bursts in neutrophils, thereby acting as inflammatory agents [10]. Therefore, it may also be possible that excessively oxidized HSA could also play a pro-oxidant role in patients undergoing dialysis. Also, it has been reported that plasma albumin is a major target for ROS in uremic patients and that the increased level of carbonyl groups is positively correlated with its oxidation, suggesting its important protective functions.

In this study we investigated the effect of haemodialysis on structural changes of albumin in comparison with healthy patients. Furthermore, the level of oxidative stress in chronic renal failure patients before, during and after regular haemodialysis was analysed. The aim of our study was to show that haemodialysis therapy leads to the plasma protein damage in relation to the decrease of low molecular weight antioxidants. Another purpose of this investigation was to evaluate how the process of haemodialysis affects the chosen blood parameters regardless of disease aetiology. The drop of antioxidants induces oxidative stress, which has been evaluated in this study by the investigation of several markers. If during haemodialysis biological materials have been damaged, we propose that it might be a therapeutic advantage to enrich dialysis fluid by the low molecular weight antioxidants. To the best of our knowledge this is the first study demonstrating albumin changes in its different regions after haemodialysis with the usage of the broad variety of fluorescent dyes.

Materials and methods

Chemicals

Fluorescence labels ANS-1-anilino-8-naphthalenesulphonate acid, 4,4′-Bis(1-anilino-8-naphtalenesulfonic acid), fluorescein isothiocyanate (FITC), pirenomaleimid, piren were purchased from Molecular Probes (USA). Tempamine (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl), DTNB [5,5′-dithio-bis (2-nitrobenzoic acid)], DNPH (2,4-dinitrofenylohydrazine), NBT (nitroblue tetrazolium) and Trolox were obtained from

Sigma (St. Louis, MO) and xylenol orange from ICN. All other chemicals were analytical grade products from POCh (Gliwice, Poland).

Subjects

The study population consisted of 10 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 eight patients were with glomerulonephritis, one with diabetic nephropathy and one with polycystic kidney disease. Patients were dialysed with the use of cuprophan dialysers DIACAP CE 1600. The mean age of recruited patients was 58 [$\pm$11] years. All patients received erythropoietin. The control group of healthy subjects was recruited among volunteers of the Outpatient Center of Medical University in Lodz. The mean age of control group was 46 [$\pm$15] years.

Venous blood samples for albumin investigations were collected before and after dialysis. For analysis of the level of oxidative stress parameters the blood samples were additionally taken during haemodialysis (at the 20th and at the 60th min). After blood centrifugation the plasma was used for further investigations. Red blood cells were washed with saline phosphate buffer pH 7.4. Erythrocyte membranes were prepared by hypotonic lysis using the method of Dodge et al. [15].

Ethical approval was obtained from the Medical University of Lodz. All subjects signed an Informed Consent Form prior to participation.

Albumin isolation

HAS samples were isolated using 60% ammonium sulphate $(NH_4)_2SO_4$. The ratio of plasma and $(NH_4)_2SO_4$ was 1:1.4. Then plasma was centrifuged at 3000–5000 g/min for 10 min. To eliminate ammonium sulphate, supernatant with albumin was dialysed against deionized water twice. The third dialysis was performed against 0.9% NaCl in 5 mmol/l phosphate buffer, pH 7.4. Every dialysis lasted for 4 h. The purification of HSA samples was further continued on gel filtration (Sephadex G-25 Superfine). Albumin fractions were collected into 20 samples, for which absorbance at 280 nm was measured. The fraction with the highest absorbance was taken for further investigations. Protein concentration was estimated according to the Lowry et al.'s [16] assay. Absorbance was red at 750 nm. Protein concentration was estimated from the standard curve and its amount was expressed in μg.

Fluorescent labelling and measurements

Changes in the albumin modified by dialysis process were investigated by spectrofluorometric method. Measurements of fluorescent intensity were used in the investigations of the microenvironment in the region labelled by fluorescent dye. Albumin in the concentration of 1 mg/ml was taken for spectrofluorometric analysis.

Five different fluorescent labels were applied for which fluorescent intensity and anisotropy was measured:

- ANS (1-anilino-8-naphthalenesulphonate acid) binds with albumin in two sites: in sub-domain IIA and IIIA through the hydrophobic interactions. ANS fluorescence increases in a more hydrophobic environment [17–19]. ANS in the final concentration of 100 μmol/l was added to the solution of 0.2 mg/ml albumin in PBS. After 15 min of incubation fluorescent intensity was measured in the samples.
- Bis-ANS 4,4′-Bis(1-anilino-8-naphtalenesulfonic acid) binds with proteins in the hydrophobic regions with affinity $\sim$10–100-times higher than ANS. Bis-ANS in the final concentration of 5 μmol/l was added to the solution of 0.2 mg/ml albumin in PBS. After 15 min of incubation fluorescent intensity was measured in the samples.
- Piren localizes to the hydrophobic regions. Label in tetrahydrofuran in the final concentration of 2.5 μmol/l was added to the solution of 0.1 mg/ml albumin in PBS. After 30 min of incubation fluorescent intensity was measured in the samples.
- In physiological pH, pirenomaleimid binds with proteins by thiol groups leading to the tioeters formation. It interacts with albumin through the thiol group of cysteine in the 34th position. Pirenomaleimide (in tetrahydrofuran) in the final concentration of 1 μmol/l was added to the solution of 0.1 mg/ml albumin in PBS. After 30 min of incubation fluorescent intensity was measured in the samples.
- Fluorescein isothiocyanate (FITC)–isomer I in neutral pH binds with free a-amino groups of proteins (N-terminus). FITC in the final concentration of 10 μmol/l was added to the solution of 2 mg/ml albumin in carbonate buffer pH 9.2. After 15 min of incubation samples were dialysed for 1 h against PBS and then fluorescent intensity was measured.

Excitation and emission wavelengths for which measurements were performed are presented in Table I.

Analysis of oxidative stress markers in blood plasma

Plasma thiols. Plasma thiol groups were measured by the method of Ellman [20]; 100 μl DTNB [5,5′-dithio-bis (2-nitrobenzoic acid)] from a 10 mmol/l stock solution was added to a 50 μl sample in 1 ml of a 10 mmol/l phosphate buffer, pH 8.0, containing SDS. Samples were incubated for 1 h at 37°C. Thiols react with DTNB to form anions which

Table I. Emission and excitation wavelengths for different fluorescent probes.

Label	Excitation (nm)	Emission (nm)
ANS	360	466
bis-ANS	395	500
FITC	494	518
Pirenomaleimid	340	373
Piren	349	397

have a strong yellow colour and are optically active at 412 nm. The basal optical activity of the samples was measured before the addition of DTNB. The concentration of thiol groups was calculated as presence of -SH/mg protein, assuming 100% for control (healthy subjects). A similar method was applied in determination of -SH groups in isolated erythrocyte membrane.

Plasma viscosity. Plasma viscosity was determined using the EPR method with Tempamine (4-amino-2,2,6,6-tetramethylopiperidine-1-oxyl) by comparison of the relative rotational correlation time of the spin label in plasma and in distilled water [21]. The relative correlation time for Tempamine was calculated from the equation:

$$\tau_c = k \cdot w_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{-\frac{1}{2}} - 1 \right]$$

where $k = 6.5 \times 10^{-10}$ s, w_0 is the width of the spectrum middle line, h_0 is the height of the spectrum middle line and h_{-1} is the height of the spectrum low-field line.

Plasma viscosity was calculated from the equation:

$$\eta_{PLASMA} = \left(\frac{\tau_{C(PLASMA)}}{\tau_{C(H_2O)}} \right) \cdot \eta_{H_2O}$$

where η_{PLASMA} and η_{H2O} are viscosity of plasma and water, respectively ($\eta_{\mathrm{H2O}} = 1$ cP).

EPR measurements were performed at room temperature with a Bruker ESP 300E (X-band) spectrometer operating at the microwave frequency of 9.73 GHz using the following settings: centre field set at 3480 G, range at 80 G, with a 100 Hz modulation frequency and a modulation amplitude of 1.0 G.

Antioxidant plasma capacity. Antioxidant plasma capacity was determined by the spectrophotometric method using DPPH (1,1-diphenyl-2-picrylhydrazyl). Sample absorbance was measured at 517 nm. Suppression of colour intensity was proportional to antioxidant content in plasma. A calibration curve was prepared from different Trolox concentrations in the range of 0–1000 μM [22].

Glycosylated plasma proteins. Glycosylated plasma proteins were analysed with a nitroblue tetrazolium salt (NBT) [23]; 20 μl of plasma was added to 980 μl of 100 mmol/l carbohydrate buffer (pH 10.3) containing NBT (5 mmol/l). The reduction of NBT to its formazan form, which is proportional to the concentration of glycosylated proteins in plasma, was quantified by measuring changes in NBT absorbance at 530 nm between the 10th and the 20th min of the reaction at 40°C. The results are presented as percentage changes in comparison to control (100%).

Carbonyl compounds. Carbonyl content was estimated by incubating 10 μl of plasma with 1 ml of 0.2% DNPH in 2.5 mol/l HCl for 1 h at room temperature. After the reaction, proteins were precipitated with 20% trichloroacetic acid (TCA) and centrifuged. The precipitate was washed three times with ethyl acetate (1:1), dissolved in 6 mol/l guanidine hydrochloride and absorbance at 360 nm was recorded. The results were calculated as μmol carbonyl/mg protein using the molar absorbance coefficient $22\ 000 \times \mathrm{mol}^{-1} \times \mathrm{cm}^{-1}$.

Hydroperoxide measurement. For the determination of plasma hydroperoxides, xylenol orange was employed. The reaction is based on the rapid oxidation of Fe(II) to Fe(III) in the presence of peroxides [24]. The reaction of Fe(III) with xylenol orange yields a violet-coloured complex, which is quantified spectrophotometrically by determining its absorbance at 560 nm using a standard curve.

Statistical analysis

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

Results

In order to explain observed oxidative changes in plasma albumin the oxidative stress-related markers in plasma have been investigated before and after haemodialysis as well as during the treatment. Obtained results have been compared to the control samples. From this we can conclude that oxidative stress-related changes are induced by developed pathogenesis and that they are additionally accelerated by the process of haemodialysis. Analysis at 20 min of haemodialysis was motivated by previously reported results about an increase in the production of superoxide anion and hydroxyl radical [4,5]. 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 [25]. However, the 60 min

of ongoing haemodialysis was chosen because at this time of treatment the highest amount of retained toxins is removed. 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.

Albumin's properties were investigated with the usage of five various fluorescent labels, which are attached to different regions. The changes were investigated before and after haemodialysis and were compared with control samples taken from healthy patients (Table II).

In the case of ANS, the statistically significant decrease of fluorescent intensity was observed after HD ($p < 0.05$) compared to control. Those changes can be the result of high concentration of metabolic products, which inhibit binding regions in albumin (I and II sub-domain, which are also involved in ANS binding) or decrease of hydrophobicity of this environment. However, significant increase of fluorescent intensity before ($p < 0.01$) and after ($p < 0.005$) HD was observed for bis-ANS in comparison with control group. Both ANS as well as bis-ANS bind to the hydrophobic region, but these two dyes present different affinity to the same regions. The contradictory results can be explained by the fact that bis-ANS binds 100-times stronger to the hydrophobic regions than ANS. It is also possible that both dyes can react with different regions of albumin molecules. For instance, bis-ANS bound to mucin molecule, while ANS did not bind with this glycoprotein [26].

A statistically significant decrease of fluorescent intensity before and after HD ($p < 0.0002$) was observed for piren in comparison with the control group. This result may explain the decrease of hydrophobicity in the nearest environment of the label and also the changes in the albumin structure (III structure).

Unexpected results were detected for pirenmaleimide label. Statistically significant increase of fluorescent intensity was observed. This result correlates with the increase of the number of -SH groups in CRF patients undergoing haemodialysis in plasma (28% increase before and 40% after HD).

For albumin labelled with FITC the decrease of fluorescent intensity was observed after HD ($p < 0.002$). It can be explained by the generated ROS, which changes microenvironment neighbouring the amine groups of lysine residue during haemodialysis. Moreover, because of a very low level of antioxidant enzymes in cytoplasm, albumin as a main protein becomes the first target for ROS attack.

Total plasma levels of thiol groups as numerous oxidative stress markers in CRF patients before, during and after haemodialysis are depicted in Table III. Before and during haemodialysis (20, 60 min of session), an increase in the level of -SH groups was insignificant, but at the end of haemodialysis the level of -SH groups was significantly elevated (8.59 ± 0.7 μmol/mg protein vs 11.17 ± 2.2 μmol/mg protein, $p < 0.05$) compared with the healthy controls. To estimate the level of ROS generated during haemodialysis the following oxidative stress markers have been analysed before, during and after haemodialysis.

The level of thiol groups in erythrocyte membrane was also estimated in CRF patients before and after haemodialysis (Figure 1). The concentration of -SH groups was significantly lower in CRF patients than in control subjects (65.73 nmol/mg in the proteins of CRF patients vs control of 92.91 nmol/mg, $p < 0.001$). A decrease in this parameter was observed during and at the end of haemodialysis.

Plasma viscosity was determined by comparison of Tempamine spin label motion in plasma with its

Table II. Fluorescence intensity for ANS, bis-ANS, pirenmaleimid, FITC piren in the presence of healthy (control) albumin and from CRF patients.

	Fluorescence intensity		
Fluorescence label	Control	Before HD	After HD
ANS	121.962 ± 27.619 $n = 15$	101.318 ± 11.899 $n = 10$	97.647 ± 12.613 $n = 9$ $p < 0.05$
bis-ANS	203.102 ± 26.922 $n = 10$	261.828 ± 37.595 $n = 10$ $p < 0.01$	268.809 ± 32.502 $n = 9$ $p < 0.005$
Piren	20.264 ± 1.356 $n = 9$	12.868 ± 2.278 $n = 10$ $p < 0.0002$	12.725 ± 2.567 $n = 9$ $p < 0.0002$
Pirenmaleimid	28.23 ± 5.851 $n = 17$	36.167 ± 7.596 $n = 10$ $p < 0.05$	39.583 ± 6.115 $n = 9$ $p < 0.005$
FITC	903.382 ± 7.106 $n = 7$	913.023 ± 14.695 $n = 10$	872.871 ± 17.162 $n = 9$ $p < 0.002$

Table III. Thiol group concentrations, plasma viscosity and antioxidant plasma capacity of control and CRF patients before, during and after haemodialysis.

Parameter	Control	Before HD	20 min HD	60 min HD	After HD
–SH [mol/mg protein]	8.59 ± 0.7 $n = 9$	9.56 ± 2.5 $n = 9$	9.31 ± 1.6 $n = 9$	9.58 ± 1.8 $n = 9$	11.17 ± 2.2 $n = 9$ $p < 0.05$
Plasma viscosity, η [cP]	1.54 ± 0.20 $n = 11$	1.52 ± 0.24 $n = 11$	1.63 ± 0.13 $n = 11$	1.69 ± 0.14 $n = 11$	1.69 ± 0.14 $n = 11$
Antioxidant plasma capacity [%]	100 ± 22 $n = 9$	367 ± 78 $n = 9$ $p < 0.0002$	326 ± 78 $n = 9$ $p < 0.0002$	294 ± 64 $n = 9$ $p < 0.0002$	292 ± 108 $n = 9$ $p < 0.0002$

motion in water [21]. An increasing tendency of plasma viscosity in patients before, at 20 min and 60 min and after haemodialysis was observed (Table III) and was correlated with the increase in protein concentration (data not shown but available upon request).

DPPH is a stable radical, which is often used for determination of total antioxidant capacity of plasma which leads to fading of its colour. This experiment was aimed to show the changes of total plasma antioxidants level together with uric acid, which is $\sim$2–2.5-fold higher in CRF patients than in healthy subjects.

This method showed that the antioxidant capacity of the plasma of CRF patients was much higher (3–3.5-fold) than in healthy volunteers. During a haemodialysis session, a 3.7–2.9-fold decrease in the reducing potential of plasma was observed in the blood of CRF patients as compared to control value (Table III).

The level of carbonyl groups in the plasma of CRF patients was 3-fold higher (2.27 ± 0.2 μmol/l) in comparison to healthy donors (0.67 ± 0.07 μmol/l). The amount of carbonyl compounds in plasma was significantly higher at the end of haemodialysis (2.94 ± 0.12 μmol/l) than before it (2.27 ± 0.2 μmol/l) ($p < 0.0002$) (Figure 2).

Figure 3 demonstrates the level of glycosylated proteins (GP) in control and CRF patients before and after haemodialysis. The amount of GP was significantly higher ($p < 0.0005$) in the plasma of CRF patients before haemodialysis (0.068 ± 0.013) and after haemodialysis (0.072 ± 0.016) than in healthy subjects (control 0.043 ± 0.008). However, there was an insignificant increase in plasma GP after haemodialysis.

The level of plasma hydroperoxides in chronic renal failure patients (5.99 ± 0.3 μmol/l) was also significantly higher ($p < 0.0002$) than in healthy volunteers (4.93 ± 0.26 μmol/l) (Figure 4). A significant increase ($p < 0.05$) in the concentration of plasma hydroperoxides (6.48 ± 0.48 μmol/l) was observed after haemodialysis sessions in comparison with their concentration before haemodialysis (Figure 4).

Discussion

The aim of this study was investigation of HSA properties and its structural changes after modification induced *in vivo* among patients with CRF—regardless of disease aetiology—who underwent haemodialysis. We also evaluated how the process of haemodialysis affects the chosen blood parameters. Application of different fluorescent dyes allowed us to investigate different regions of albumin molecules. As markers of

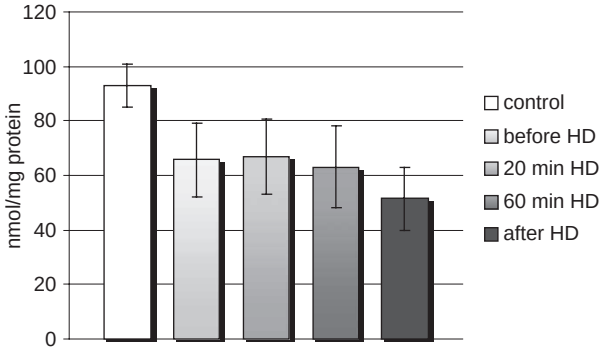

Figure 1. The level of thiol groups in control and CRF patient erythrocyte membranes before, during and after haemodialysis. Significant differences were detected in erythrocyte membranes between control and before HD and at 20 min of HD ($p < 0.001$), between control and at 60 min of HD ($p < 0.0005$) and between control and after HD ($p < 0.0002$).

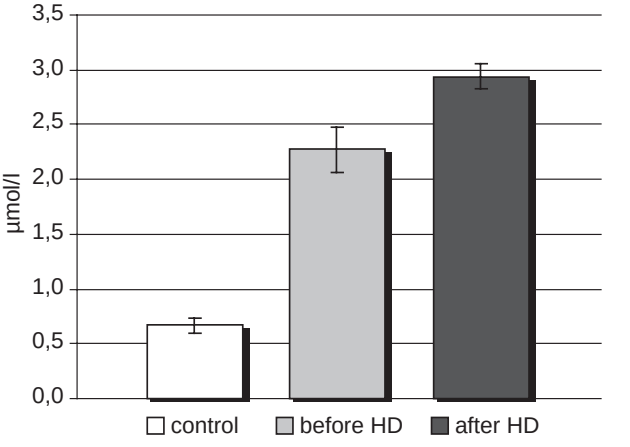

Figure 2. Formation of plasma protein carbonyls in chronic renal failure patients in comparison to healthy donors ($n = 9$). Significant differences were detected in protein carbonyls between control and before HD ($p < 0.0002$), between control and after HD ($p < 0.0002$) and before HD and after HD ($p < 0.0002$).

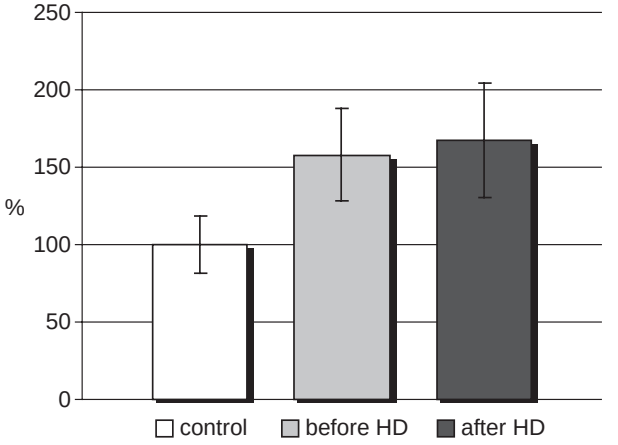

Figure 3. Levels of plasma glycosylated proteins in chronic renal failure and control subjects ($n=10$). Significant differences were detected in glycosylated protein between control and before HD ($p<0.0005$) and between control and after HD ($p<0.0002$).

oxidative modification the total protein thiol (P-SH) groups, carbonyl derivatives (CRD, carbonyl groups) and hydroperoxide were estimated in plasma. Additionally, we investigated plasma viscosity and antioxidant capacity of the plasma in CRF patients.

It has been shown that the generation of reactive oxygen species by activated neutrophils plays a key role in oxidation of lipids and proteins in erythrocyte plasma membrane [4,5,8,27–30]. In renal patients, injury of blood components can be induced directly by ROS, which oxidize -SH, produce carbonyl derivatives as well as form covalent bonds between tyrosine residues. Urea, in turn, leads to the carbamylation of proteins, peptides, glycoproteins and polysacharydes [12,31–34], which are more sensitive to oxidation by reactive oxygen species [35]. However, glucose triggers the process of glycosylation.

Thus, imbalance between ROS production and their detoxication is mainly responsible for damage of

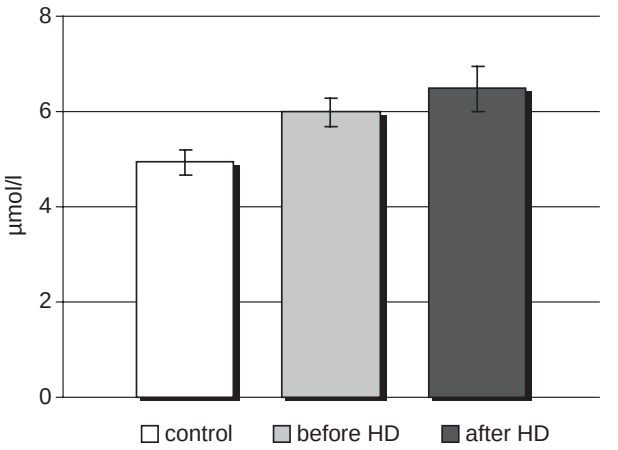

Figure 4. Plasma hydroperoxides in control and chronic renal failure patients ($n=9$). Significant differences were detected in plasma hydroperoxides between control and before HD ($p<0.0002$), between control and after HD ($p<0.0002$) and before HD and after HD ($p<0.05$).

blood components in CRF patients. That is why antioxidants in plasma play a key role in the protection of plasma components as well as cells against ROS attack in the pathogenesis of chronic renal failure of haemodialysed patients.

We have shown that carbamylated proteins are more sensitive to the oxidation process. Our results demonstrate that in patients with CRF, albumin is significantly altered before and after haemodialysis, suggesting that carbamylation and induced oxidative stress play an important role in renal pathogenesis. In the case of albumin these alterations might lead to changes in transport and antioxidant functions of the protein.

Haemodialysis significantly decreased fluorescent intensity after ANS labelling and the decreased level of binding residues in albumin (Site I and Site II to which ANS binds) were described. It can be caused by binding of metabolites to the same residues to which ANS binds or/and decrease of electrostatic interaction protein-ligand as a consequence of conformational changes of protein in this microregion. Generally, ANS exhibits electrostatic interaction with proteins and conformational changes in albumin are responsible for decreased interaction between ANS and investigated protein. The decrease of fluorescent intensity after HD for ANS can be additionally related to the weaker affinity of the dye to the hydrophobic binding centre. Furthermore, in the case of ANS there was a transition of the protein from partially folded to the unfolded protein.

Analysis with bis-ANS, which generally binds to the hydrophobic but probably other regions in albumin as well, showed that haemodialysis does not have an additional impact on conformational changes in this protein region. More molecules of this label interact with conformationally changed protein due to the development of uremic pathogenesis. Moreover, contradictory results obtained for ANS and bis-ANS can be explained by $\sim$100-times higher affinity of bis-ANS to hydrophobic regions [36] or interaction of these dyes with other regions of albumin molecule. It has been shown that ANS did not interact with mucin, while bis-ANS bound very strongly [26].

Similarly, fluorescence intensity decreased after albumin labelling with piren, which binds in hydrophobic regions of albumin and explains changes in III structure. Our results show that conformational changes of investigated albumin are less accessible for piren, which can be connected with partial loss of hydrophobicity in this region of albumin molecules. This suggests that the generated ROS change conformational structure of albumin, which directly influences hydrophobic region accessibility for other molecules, such as piren. Due to conformational changes, modified HSA does not have antioxidative properties in plasma. Our results depict that haemo-

dialysis does not significantly deepen observed changes. However, the most important impact of ROS action was driven by the development of renal pathogenesis.

Our investigation with pirenmaleimide shows the increase of fluorescence, which was related to the increase of hydrophobic properties of label micro-enviroment as well as an increase in –SH groups. Additionally, considering that albumin possesses only one free -SH group, one molecule of pirenmaleimide interacts with this group leading to the elevation of fluorescent intensity dictated by the increase of hydrophobic properties in the investigated albumin region. Moreover, changes in albumin molecules can also exhibit internal –SH groups, which formed –S-S-bridges. This process was observed during albumin denaturation. The formation of carbonyl groups in plasma albumin as well as Cys-34 with free thiol group was described [8]. It was also detected that the carbomylation of albumin accounts for most of the plasma protein oxidation in HD [37,38]. In those patients oxidative modifications of the most important protein antioxidant such as albumin appear to be more extensive.

Haemodialysis also significantly decreased fluorescent intensity after FITS labelling, which suggests alterations in the microregion of labelled lysine residues. A significant drop of fluorescence intensity is related to the conformational changes of albumin structure in the microenvironment.

Inducing oxidative stress by exposing blood to membrane dialyser and chronic mechanical trauma during haemodialysis provoked changes in conformational and functional properties of plasma albumin, which resulted in the loss of its biological functions. Generally we have shown changes in the structure of albumin from CRF patients in comparison to control. These results clearly indicate that haemodialysis enhanced alterations in albumin molecules. They also confirm our earliest findings that partially carbamylated/oxidized protein is easy apt for oxidation during haemodialysis. Moreover, plasma biochemical properties alterations of the albumin structure and accelerated ROS production can be responsible for the development of other clinical complication like inflammation, malnutrition, atherosclerosis syndrome in CRF patients and/or patients undergoing haemodialysis.

In plasma amino acids in protein are susceptible to modifications induced by ROS. Especially cysteine, lysine, arginine and histidine are extremely vulnerable to oxidative modifications [39] and alterations in these residues led to conformational changes of albumin in CRF patients. We showed that in plasma the -SH groups increase with parallel release of the GSH and Hb from disrupted RBC. Furthermore, consistently with the results obtained by Himmelfarb [40], also significant increase of –SH groups in HD patients was detected.

An opposite result to plasma –SH groups was found in the case of erythrocyte membrane, where the decrease of the –SH level was observed. Thiols are very sensitive to oxidation during oxidative stress, which leads to thiol bridges formation or SO_x residues. In the case of erythrocyte membrane oxidative stress definitely took place, which is correlated with the decrease of the level of antioxidants enzymes as well as with ROS production [25].

In CRF patients we observed significant influence of HD therapy on the oxidative stress-related markers. Thus, oxidative burst with neutrophiles and Hb as catalyser of ROS reaction induce oxidative stress in the plasma.

It can be expected that changes in plasma viscosity result from haemodialysis treatment. By determining the viscosity of plasma and total level of proteins before and at the end of haemodialysis using electron paramagnetic resonance we showed the tendency of an increase in viscosity during and after haemodialysis. This result was correlated with an increase in the total level of proteins in plasma. Elevation of the total level of proteins may be also related to the release of haemoglobin and other proteins from disrupted erythrocytes. Moreover, upon haemodialysis treatment plasma was dehydrated, which resulted in the increased protein concentration as well as in plasma viscosity. Obtained results confirm other findings [9,41–44].

Additionally, plasma antioxidant capacity was determined using the DPPH radical. The antioxidant capacity of plasma in haemodialysed patients was more that 3.5-fold higher than that of the control group. During haemodialysis the antioxidant potential decreases, but after haemodialysis it is much higher than in healthy volunteers.

The 3.5-fold increase of antioxidant capacity of plasma before haemodialysis session estimated by DPPH is in accordance with other methods. An $\sim$3-fold higher antioxidant capacity of plasma was also observed before haemodialysis using the FRAP method [24,28,45–48], but after haemodialysis it recovered to control levels. Plasma antioxidant capacity depends on the concentration of low molecular weight antioxidants such as uric acid, ascorbic acid and tocopherols. In the plasma of chronic renal failure patients, the concentration of uric acid was 2-3-fold higher than in healthy controls, but the concentrations of ascorbic acid or α-tocopherol were similar [9,28,46,49]. During haemodialysis uric acid and other low molecular weight antioxidants are removed from blood and then total antioxidant capacity decreases.

We also showed that the level of carbonyl groups increases in CRF patients and is additionally

accelerated upon HD as a negative effect of induced oxidative stress.

Proteins are modified directly by ROS or indirectly with reactive carbonyl compounds formed by oxidation of carbohydrates and lipids. Precursors of RCD are elevated in circulation of uremic patients. Also concentration of advanced glycation and lipid peroxidation is increased in plasma and in matrix in comparison to healthy subjects [50]. The consequences of such changes can be impaired enzymatic activity, damage of cellular membrane and alterations in cellular functions. In this study the level of carbonyl compounds was determined to be 3-fold higher in chronic renal failure patients than that in healthy controls. The level of carbonyls increased during haemodialysis and at its end it was 4-fold higher than in control blood. An increase in plasma carbonyl compounds was described in the literature [38,51]. These results also indicate oxidative damage of plasma proteins during haemodialysis. It has been reported that free radicals production and a decrease in antioxidants may modulate the formation of early glycated proteins *in vivo* [52]. Another possibility of RCD increased level can be related to the lower rate of protein degradation [53,54]. However, results obtained by Mimić-Oka et al. [50] indicate that the accumulation of RCD in CRF did not depend on the amount of plasma proteins. It confirms the hypothesis that ureamia appears to be in a state of carbonyl overload with damaging proteins.

Chronic renal disease often accompanies a higher level of glucose in the blood and carbonyl compounds can be produced by non-enzymatic glycation of proteins. In this study a significantly higher level of glycated proteins was indicated in CRF patients and increasing tendency after haemodialysis was observed. In physiological conditions, the kidney contributes to the maintenance of glycaemic levels and the loss of these functions leads to the glycaemic disorders present in these patients. In non-diabetic CRF patients the abnormal glucose metabolism is responsible for the development of hyperlipidaemia. These alterations might contribute to the increase of cardiovascular risk in these patients [49].

Similarly, the level of hydroperoxides increases in CRF patients due to alterations in antioxidants system and HD deepen the changes. These markers describe direct effect of oxidative stress induced by HD.

In conclusion, conformational changes of HSA from CRF patients are triggered by ROS (protein oxidation), urea (protein carbamylation) or glucose (protein glycosylation). These changes in plasma are mainly induced by oxidative stress, which was reflected in the increase of the level of hydroperoxides and carbonyls. However, in the case of RBC it is possible that, due to the decrease of the level of –SH groups in cellular membrane, either carbamylation effect was induced because of the increased level of urea in CRF patients or oxidative stress was induced in consequence of decreased antioxidants.

Based on the obtained results we propose that, regardless of CRF aetiology, all patients undergoing haemodialysis should be supplied by antioxidants therapy. It might be advantageous to enrich dialytic fluid with low molecular mass antioxidants. It would help to keep balance in the antioxidants concentration between plasma and dialytic fluids. Therefore, it would protect CRF patients against various side-effects induced by haemodialysis treatment.

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