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QUARTERLY

Changes in red blood cell membrane structure in patients with chronic renal failure*

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The properties of red blood cell membranes in patients with chronic renal failure were investigated using electron paramagnetic resonance spectroseopy. Using spin traps, 5,5-dimethylpirroline-1-oxide and *N*-tert-butyl-α-phenylnitrone, we found generation of hydroxyl radicals in the blood of patients with chronic renal failure after 20 min of regular hemodialysis.

The physical state of membrane proteins and membrane osmotic fragility and reductive properties of red blood cells were studied. The increase in the relative correlation time of 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl indicates the immobilization of membrane protein molecules in erythrocytes of chronic renal failure patients. The decrease in membrane protein mobility was observed in whole blood incubated with tert-butylhydroperoxide, regardless of the presence of iron. We found that the addition of ferrous ions did not aggravate profound changes in membrane proteins induced with tert-butylhydroperoxide.

We also demonstrated higher osmotic fragility of erythrocytes in the patients with renal failure as compared to normal subjects.

These alterations in membrane structure of red blood cells in hemodialysed patients suggest that hydroxyl radicals generated during hemodialysis can play an important role in the oxidative mechanism of erythrocyte damage.

The reduction of an oxygen molecule leads to generation of its reactive forms, i.e. superoxide radical, hydrogen peroxide and hydroxyl radical. Reactive oxygen species are permanently formed in a human body by activated phagocytes during chemical side reactions. However, in a normal organism there exists a balance between reactive oxygen species formation and their detoxication. Red blood cells (RBCs) exist in an environment in which they are permanently exposed to both intracellular and extracellular

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Abbreviations: DMPO, 5,5-dimethylpirroline-1-oxide; DMSO, dimethylsulfoxide; EPR, electron paramagnetic resonance; POBN, *N*-tert-α-phenylnitrone; RBCs, red blood cells; t-BuOOH, tert-butylhydroperoxide.

sources of free radicals. In erythrocytes the constant conversion of hemoglobin into methemoglobin and superoxide radical generation take place [1, 2]. In addition to RBCs, granulocytes and macrophages, as well as other metabolically active cells, generate active oxygen species [3–5]. Neutrophils can use hydrogen peroxide produced by the dismutation of superoxide anion radicals and oxidize chloride ions by myeloperoxidase into hypochlorous acid, another oxygenated agent [5, 6]. These toxic oxygen species can diffuse into extracellular environment and many of them have been found to interact with RBCs [7, 8].

Imbalance between free radical formation and their detoxication leads to cell damage.

The lifespan of RBCs in patients with chronic renal failure is known to be much shorter than in healthy subjects. In chronic renal failure patients the intracellular defence system of RBCs against toxic oxygen species is disturbed, the catalase activity being decreased [9] and the content of glutathione diminished [10, 11]. On the other hand, opposite results were reported on superoxide dismutase [12–15] and glutathione peroxidase activities in erythrocytes [11–13]. Lipid peroxidation in plasma membranes was also observed in RBCs of patients with chronic renal failure [14–17]. In the blood of these patients there is a higher concentration of iron and aluminium, two catalysts of free radical reactions [18]. Under these conditions, RBCs can be easily damaged by toxic oxygen forms.

In this study we made an attempt to confirm the generation of toxic oxygen species as well as their effects on the structure of RBCs. We also endeavored to estimate the relationship between oxidative damage of membrane proteins and reactive oxygen species generation in erythrocytes using spin labeling and spin trapping methods.

MATERIALS AND METHODS

Chemicals. 5,5-Dimethylpirroline-1-oxide (DMPO), L-tert-a-phenylnitrone (POBN) and tert-butylhydroperoxide (t-BuOOH) were from Sigma (St. Louis, MO, U.S.A.); 4-(2-iodoacetamide)-2,2,6,6-tetramethylpipe- ridine-1-oxyl (iodoacetamide spin label) was prepared according to McConnell *et al.* [19]. All other chemicals were analytical grade products from POCh (Gliwice, Poland).

Red blood cells and red blood cell ghosts preparation. Human blood anticoagulated with heparin was obtained from six patients with chronic renal failure before and after 20 min and 60 min of regular hemodialysis. All patients were dialysed using cuprophane dialysers Lundia 10 3N (Gambro) and acetate buffer. Control blood was taken from six healthy volunteers. Blood was centrifuged ($3000 \times g$ for 5 min at $4^{o}C$) and washed twice with 0.9% NaCl and once with phosphate buffered saline, pH 7.4. RBCs were resuspended in the above buffer at a hematocrit of 50%.

The red blood cell ghosts were prepared by hypotonic lysis according to the Dodge *et al.* [20].

Spin trapping. Free radicals formed during hemodialysis in serum and red blood cell suspension were trapped using POBN and DMPO. Samples of blood were withdrawn before and after 20 min of hemodialysis and centrifuged. RBCs were separated from serum and washed with cold 0.9% NaCl. To each sample containing 80 µl of serum or packed RBCs 20 µl of 0.1 M of POBN in DMSO was added. The Fenton reaction was carried out according to Burkitt *et al.* [21].

tert-Butylhydroperoxide treatment of red blood cells. Control blood was treated at room temperature for 1 h with 0.1 mM t-butylhydroperoxide in the absence or presence of 0.1 mM $FeSO_4$. Following the treatment, RBCs were isolated, washed twice with 0.9% NaCl and once with phosphate buffered saline, and erythrocyte ghosts were prepared as described above.

Spin labeling of red blood cell ghosts. Red blood cell ghosts were labeled with the iodoacetamide spin label and incubated for 1 h at room temperature. The unbound spin label was removed by several washings with phosphate buffered saline until the EPR signal in the supernatant disappeared. The motion of the labeled proteins in red blood cell membranes, can be estimated by the calcula-

tion of the rotational correlation time from the equation [22, 23]:

$$\tau_c = k \cdot W_0 \cdot [(h_0/h_{-1})^{1/2} - 1]$$

where W_0, h_0 and h_{-1}, are midfield line width, midfield line height and high-field line height, respectively.

Osmotic fragility measurements. For osmotic fragility measurements stock suspensions of RBCs were added to the solutions containing various concentrations of NaCl. The absorbance of supernatant *vs* water was determined on Varian (Carry) spectrometer at 575 nm. The ratio of hemolysis was calculated from the equation:

$$H = A_x - A_c / A_{100} - A_c$$

where H is the ratio of hemolysis; A_x, absorbance of the sample; A_c, absorbance of the control, and A_{100}, absorbance after complete hemolysis.

EPR measurements. EPR spectra were recorded on Bruker ESP 300E X-band spectrometer equipped with a Stellar gas flow temperature controller (supplied by Polish Research Foundation). EPR measurements were performed at room temperature.

Statistical analysis included the calculation of means ±S.D. The significance of differences was estimated by Student's *t*-test.

RESULTS

In order to determine the suitability of the methyl radical spin-trapping method for the detection of hydroxyl radicals, some preliminary experiments were performed using a model system, in which we showed hydroxyl radical formation in the Fenton reaction, both in the buffer and in the presence of intact plasma.

EPR spectra of DMPO and POBN-adduct radicals in the model system as well as in plasma and red blood cells after 20 min hemodialysis are shown on Figs. 1 and 2. The Figures show the formation of hydroxyl radicals in the blood of patients with chronic renal failure during hemodialysis. We found spin adducts of DMPO and POBN in plasma and within red blood cells. The physical state of proteins in membranes was studied using

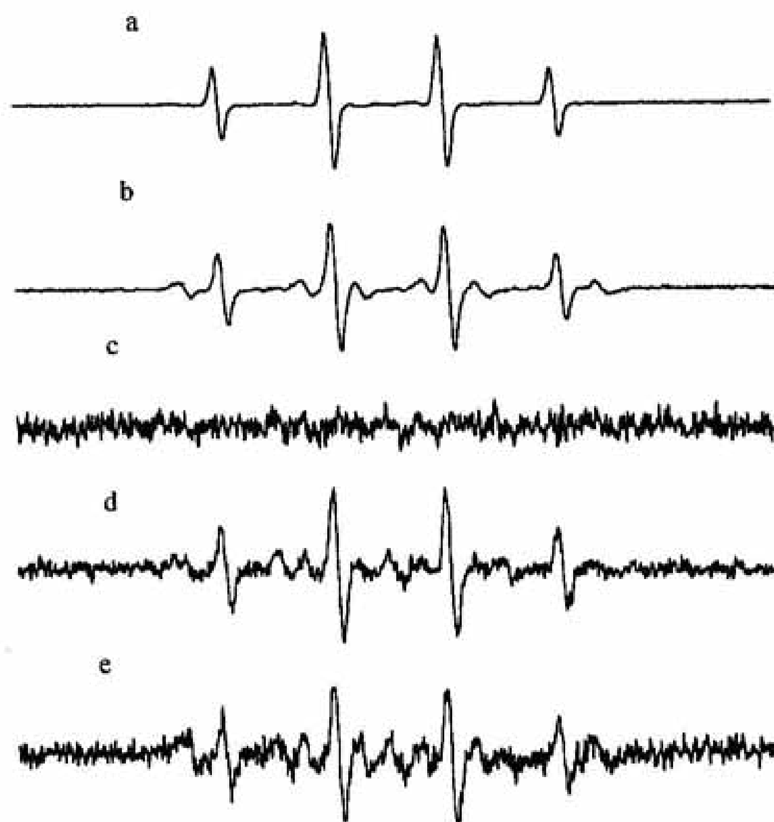

Figure 1. EPR spectra of DMPO-OH radicals formed during hemodialysis in plasma and human red blood cells.

a) Complete system (containing DMPO and Fe^{2+}/H_2O_2 (Fenton reagent)); b) DMPO + Fe^{2+}/H_2O_2 + human plasma from healthy donors; c) DMPO + plasma from chronic renal failure patients (before hemodialysis); d) DMPO + plasma (after 20 min of hemodialysis); e) DMPO + RBCs (after 20 min of hemodialysis).

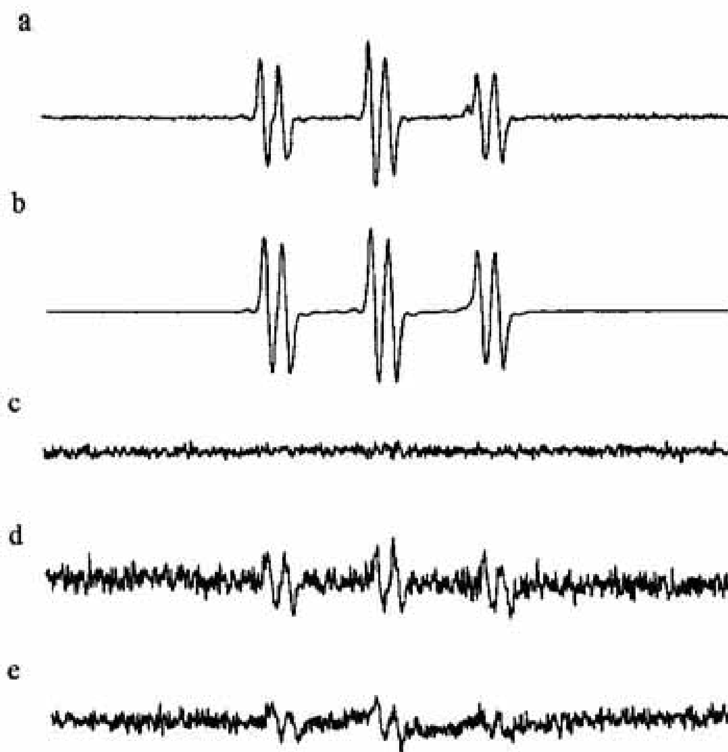

Figure 2. EPR spectra of POBN-adduct radicals formed during hemodialysis.

a) Complete system (containing POBN, DMSO and Fe^{2+}/H_2O_2 (Fenton reagent)); b) POBN, DMSO + Fe^{2+}/H_2O_2 + human plasma from healthy donors; c) POBN, DMSO + plasma (from chronic renal failure patients before hemodialysis); d) POBN, DMSO + plasma (after 20 min of hemodialysis); e) POBN, DMSO + RBCs (after 20 min of hemodialysis).

the iodoacetamide spin label, a covalently bound spin label. At neutral pH iodoacetamide reacts mainly with thiol groups of proteins [24]. Figure 3 shows EPR spectrum of iodoacetamide spin label attached to membrane proteins. The rotational correlation time (τ_c), a sensitive indicator of the physical

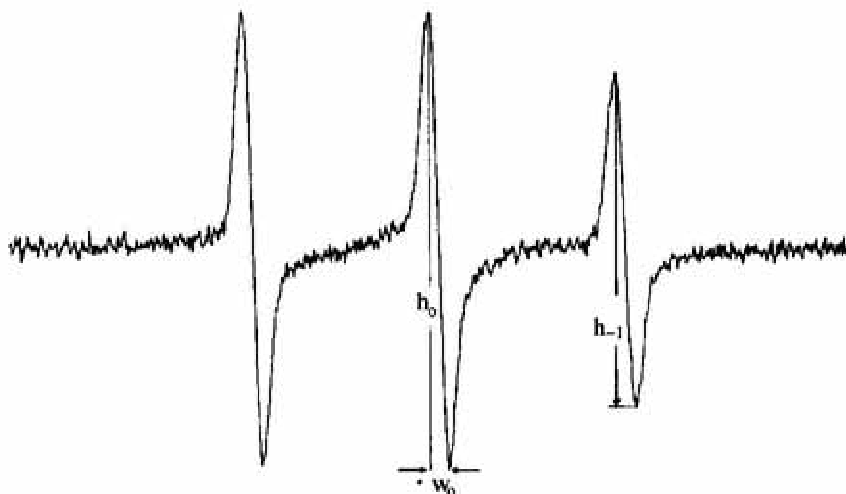

Figure 3. EPR spectrum of 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl attached to red blood cell ghosts.

state of membrane proteins [25, 26] was significantly increased (Fig. 4). This result indicates a decrease in membrane protein mobility in RBCs of chronic renal failure patients. However, the mobility of membrane proteins did not fluctuate in the course of hemodialysis. The decrease in membrane proteins mobility was also observed when RBCs were subjected to oxidative stress under *in vitro* conditions. Figure 5 shows the changes in the rotational correlation time of iodoacetamide spin label attached to membrane proteins in whole blood following its treatment with t-BuOOH. These experiments were performed in the presence or absence of ferrous ions. The changes in membrane proteins induced by t-BuOOH were similar to those induced by hemodialysis. The addition of ferrous ions had no effect on the t-BuOOH-induced damage of membrane proteins (Fig. 5).

The osmotic fragility of RBCs of chronic renal failure patients was also determined. Figure 6 presents the osmotic fragility of

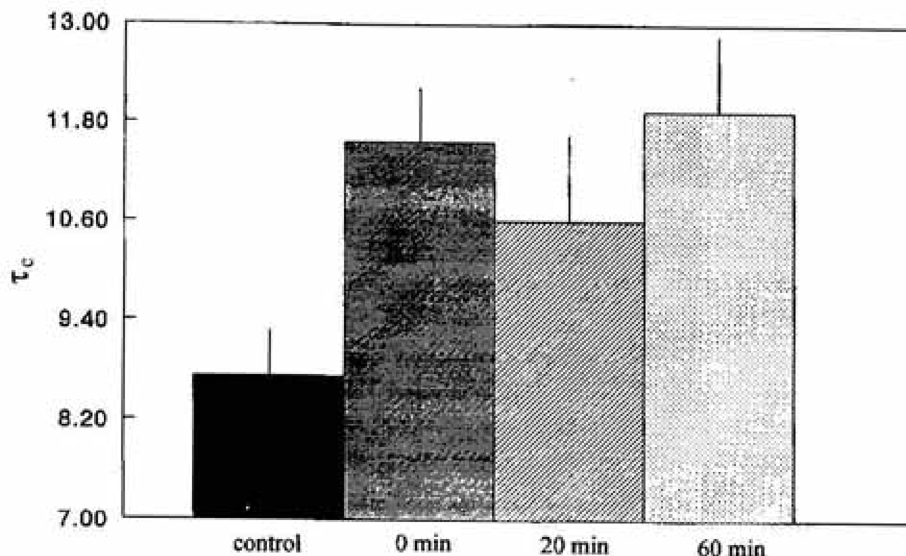

Figure 4. Changes in erythrocyte membrane proteins mobility in chronic renal failure patients visualized by iodoacetamide spin label ($P < 0.001$).

Indications at the abscissa: control, healthy donors; 0 min, chronic renal failure patients before dialysis; 20 min and 60 min, patients 20 and 60 min after dialysis, respectively.

RBCs in normal and dialysed patients (expressed as concentration of NaCl at which 50% of control and chronic renal failure RBCs were lysed). RBCs of chronic renal failure patients exhibited significantly higher osmotic fragility than control ones.

DISCUSSION

The spin trapping method is often used for short-lived free radicals such as hydroxyl and/or superoxide. In the reaction between a short-lived radical and diamagnetic spin trap a much more stable free radical adduct is formed. In this study we also applied the reaction in which hydroxyl radical reacted with DMSO producing the secondary free radical, methyl radical. In this reaction DMSO was a scavenger of hydroxyl radicals but not of superoxide radicals.

Our present study provides direct spectroscopic evidence that hydroxyl radicals can be formed during regular hemodialysis and can damage red blood cell structure. It has been shown that oxygen free radicals are generated by activated neutrophils. The contact of neutrophils with couprophane dialysis membranes enhances their oxidative metabolism. During hemodialysis higher superoxide production was also reported [10, 27]. It is known that toxic oxygen species released

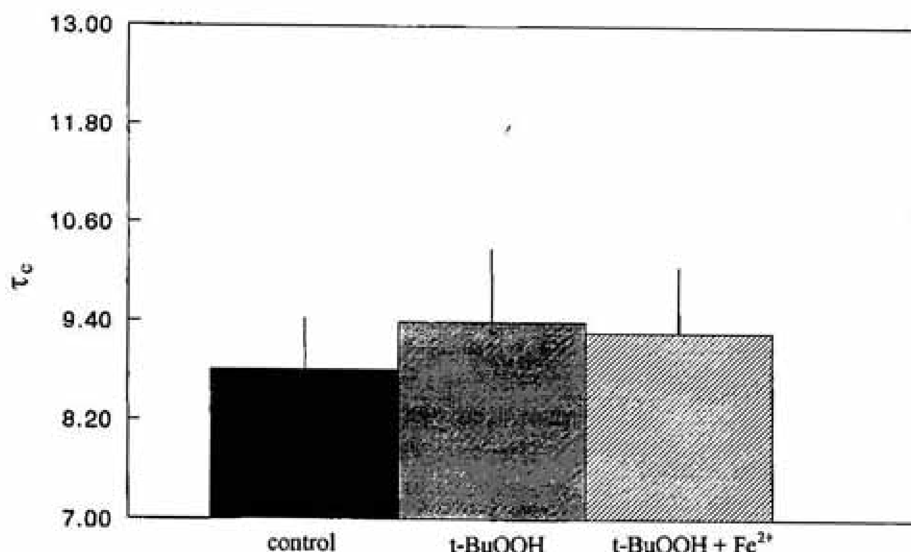

Figure 5. The effect of t-BuOOH on erythrocyte membrane proteins mobility visualized by iodoacetamide spin label (control *vs* t-BuOOH-treated erythrocytes + Fe^{2+}, $P < 0.5$).

from neutrophils can damage red blood cells. However, so far there was no direct evidence pointing to the hydroxyl radicals formation during hemodialysis. The results presented in this work clearly point to $^{\bullet}OH$ formation in the blood of patients undergoing hemodialysis. There are a few possibilities of hydroxyl radical production in the blood of hemodialysed patients (*see below*). It is of note that the blood of hemodialysed patients is overloaded with iron. On the other hand, it is commonly known that superoxide radical and hydrogen peroxide may produce hydroxyl radicals in the Haber-Weiss reaction in the presence of traces of heavy metal ions (Fe^{2+} or Cu^{2+}) [28]. In neutrophils, hydroxyl radicals can be generated *via* an alternative mechanism in the metal-catalyzed Haber-Weiss reaction. The cells release hypochlorous acid and in the presence of superoxide radicals, hydroxyl radicals can be generated [29, 30]:

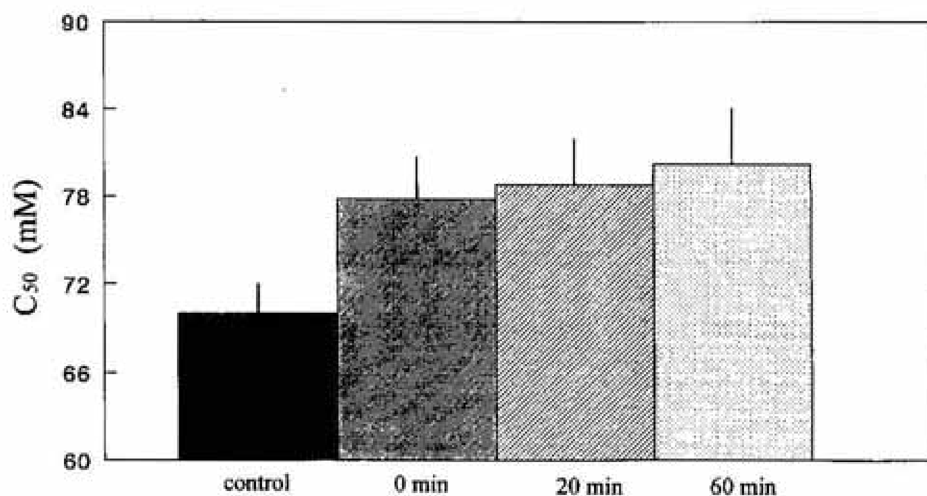

Figure 6. Osmotic fragility of RBCs in control and chronic renal failure.

Control *vs* from chronic renal failure RBCs before and during hemodialysis ($P < 0.001$). Ordinate: C_{50}, NaCl concentration at which 50% of RBCs is hemolysed. Abscissa: indications as in Fig. 4.

$$O_2^{\bullet -} + HOCl \rightarrow {}^{\bullet}OH + Cl^- + O_2$$

This reaction is analogous to the Haber-Weiss reaction, rapid and not involving metal ions, and it can be of importance in the biological activity of neutrophils [30, 31].

Another source of hydroxyl radicals is peroxynitrite, which decomposes to give some reactive species [32, 33]. This reaction is in chronic renal failure patients possible due to the higher level of nitric oxide (NO) during hemodialysis [34]. NO in the reaction with $O_2^{\bullet -}$ may produce highly cytotoxic peroxynitrite [32, 33].

Red blood cells in chronic renal failure patients have disturbed defense systems against oxygen free radicals. Hence, they are very sensitive to the damage by the reactive oxygen species. It seems that, during regular hemodialysis, RBCs are constantly damaged, mainly by $^{\bullet}OH$ radicals. This assumption is very probable because higher levels of thiobarbituric acid-reactive substances and 4-hydroxynonenal (both increased over twofold) were found in the samples after hemodialysis compared to those collected prior to hemodialysis [35]. This finding indicates that the lipid peroxidation process occurs during hemodialysis.

Hitherto, several studies on RBCs in hemodialyzed patients concerned membrane lipids. In such patients an increase of lipid peroxidation and decrease of membrane fluidity have been reported [11, 12]. In the present study we demonstrate changes in the properties of plasma membrane proteins and in the osmotic fragility of red blood cells. We found a significant decrease in membrane protein mobility in red blood cells of chronic renal failure patients, as monitored by iodoacetamide spin label. Although red blood cell membrane proteins showed irreversible alterations in their structure, the mobility of membrane proteins was not considerably changed in the course of hemodialysis. Simi-

lar changes in membrane proteins structure were found after t-BuOOH treatment of erythrocytes *in vitro*. t-BuOOH is known to damage red blood cell structure [36], and it was shown that the exposure of RBCs to this agent results in the oxidation of plasma membranes [37]. In our present study t-BuOOH induced a decrease of membrane protein mobility, which resembled that observed in chronic renal failure patients undergoing hemodialysis. It was surprising that the addition of ferrous ions did not deepen the changes in t-BuOOH-induced damage of membrane proteins, as it is known that ferrous ions are able to catalyse free radical decomposition of hydroperoxides. In the interpretation of these apparently conflicting observations we suggest that free iron might be immediately bound by membranes or complexed by serum proteins (in the case of whole blood). Our results can also indicate that hydroxyl radical formation in Haber-Weiss reaction in the blood of hemodialysed patients is probably of little importance. It seems that the increased osmotic fragility of RBCs might be a consequence of lipid peroxidation and alteration of membrane proteins.

In conclusion, the alterations in membrane structure of RBCs suggest that free radicals generated during hemodialysis play an important role in the oxidative mechanism of erythrocyte damage. Further, our observations support the view that it is predominantly the hydroxyl radicals that contribute to the altered conformation of erythrocyte membrane proteins in chronic renal failure patients.

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