Determination of erythrocyte antioxidant capacity in haemodialysis patients using electron paramagnetic resonance

Andree Klemm\textsuperscript{1}, Christine Voigt\textsuperscript{1}, Manfred Friedrich\textsuperscript{2}, Reinhard Fünfstück\textsuperscript{1}, Heide Sperschneider\textsuperscript{1}, Ernst-G. Jäger\textsuperscript{2} and Günter Stein\textsuperscript{1}

\textsuperscript{1}Department of Internal Medicine IV and \textsuperscript{2}Institute of Inorganic Chemistry, Friedrich-Schiller-University of Jena, Jena, Germany

Abstract

\textbf{Background.} The increased oxidative stress of uraemia is caused both by an increased generation of oxygen-free radicals and a decrease of antioxidative forces. There are, however, conflicting data concerning disturbances of the radical-scavenging power of red blood cells (RBCs) in uraemic patients.

\textbf{Methods.} The antioxidant capacities of the RBCs of 10 haemodialysis (HD) patients and 10 controls were examined after treatment with 0.324 mM tert-butylhydroperoxide (t-BOOH) in phosphate-buffered saline at 37°C using electron paramagnetic resonance (EPR) with 5,5-dimethylpyrroline-N-oxide (DMPO) as a spin trap and glutathione (GSH) regeneration as an indicator of hexose monophosphate shunt (HMPS) activity. EPR investigations were also done after pre-incubation with \textit{N}-ethylmaleimide (NEM) to inhibit the GSH system. Furthermore, we determined the RBC redox state in 15 HD patients and 15 controls.

\textbf{Results.} There was no difference between HD patients and controls in the elimination of t-BOOH-generated free radicals in the RBCs. A more than 20-fold increase in radical concentration was observed after GSH trapping with NEM. In this case, we found a delayed decrease of the relative radical concentration in HD patients compared with controls with a significant difference after 7 min (2.2 ± 0.26 vs 1.60 ± 0.21; \(P = 0.005\)) and after 10 min (1.82 ± 0.41 vs 0.83 ± 0.44; \(P = 0.001\)). GSH regeneration via HMPS did not differ between the RBCs of HD patients (99.5 ± 13.5 nmol/min×ml RBC) and those of the controls (94.2 ± 16.9 nmol/min×ml RBC). There were no differences in the RBC concentrations of GSH, GSSG, NADP, NADPH, and in the GSH/GSSG and NADP/NADPH ratios between HD patients and controls.

\textbf{Conclusions.} These data suggest a strong antioxidant potential in the GSH system of erythrocytes without any evidence of a disturbance in HD patients. The HMPS pathway also appears not to be impaired in the RBCs of HD patients. However, the slower radical elimination in the RBCs of HD patients after inhibition of GSH-depending radical scavengers as compared with controls indicates a defect in the antioxidant forces outside the GSH system, and could be one reason for the reduced lifespan of RBCs in HD patients.

Keywords: anaemia; antioxidant capacity; electron paramagnetic resonance spectroscopy; haemodialysis; oxygen radicals; red blood cells

Introduction

Oxidative stress is defined as a disturbance in the pro- and antioxidant balance in an organism in favour of the pro-oxidant. Oxygen-free radicals and reactive-oxygen species are involved in the pathogenesis of many clinical disorders by damaging lipids, proteins, and DNA or by altering cellular signal transduction [1]. The uraemic state and the bio-incompatibility of haemodialysis (HD) are associated with an increased oxidative stress in HD patients presumably caused by both an increased generation of oxygen-free radicals/reactive-oxygen species and decreased levels of different antioxidants [2]. Oxidative stress is thought to be the most likely explanation for the shortened lifespan of red blood cells (RBCs) in HD patients [3].

The antioxidant defenses of blood are composed of: (i) plasma antioxidants and (ii) the antioxidant capacity of the RBCs. According to Beutler’s hypothesis, RBCs can be looked upon as small detoxifying packets removing harmful substances from the plasma [4,5]. Disturbances in the antioxidant metabolism of RBCs in uraemic patients have been reported by...
[45x149]redox state in HD patients.

[45x159]of GSH with an increase in flux through the HMPS for the addition of GSH as an indicator of the catalytic activity of HMPS, which accelerate the chain reaction of lipid peroxidation. A substance initiating radical formation to investigate organic hydroperoxide frequently is used as a model. The antioxidant capacity of isolated intact erythrocytes of uraemic patients obtained from uraemic patients after antioxidant capacity in RBCs include the measurement of MDA production or the estimation of radical generation with chemiluminescence after treatment with tert-butylhydroperoxide (t-BOOH) treatment [11,12].

Electron paramagnetic resonance (EPR) with spin trapping is the only direct method of detection and identification of free radicals. Free-radical processes in RBCs have been successfully investigated by EPR [13,14]. Gwozdzinski et al. [14] using ERP, showed a decrease in membrane protein mobility of RBCs after t-BOOH treatment, and with this method were able to observe the generation of hydroxyl radicals during HD.

The aim of our study was to examine the feasibility of using EPR in the direct determination of the antioxidant capacity of isolated intact erythrocytes obtained from uraemic patients after in vitro treatment with the pro-oxidant t-BOOH. This artificial organic hydroperoxide frequently is used as a model substance initiating radical formation to investigate oxidative processes. t-BOOH easily penetrates the cell membrane and forms peroxy and alcoxyl radicals which accelerate the chain reaction of lipid peroxidation [1]. We also assessed the regeneration rate of GSH as an indicator of the catalytic activity of HMPS, for the addition of t-BOOH leads to a consumption of GSH with an increase in flux through the HMPS [15]. Furthermore, we determined the erythrocyte redox state in HD patients.

**Patients and methods**

**Patients**

We studied 15 patients (nine females, six males), with a mean age of 51 ± 16 years who had end-stage renal disease and were undergoing regular HD and 15 healthy volunteers (eight females, seven males), with a mean age of 55 ± 18 years. Informed consent was obtained from each patient, and the study was approved by the local review board. The causes of chronic renal failure were: chronic glomerulonephritis (four patients), polycystic kidney disease (three), interstitial nephritis (three), diabetic nephropathy (three) nephrosclerosis (one), and nephrophthisis (one). The mean duration of HD was 4.2 ± 2.6 years. All patients were being dialysed three times weekly for 3.5–5 h. The membranes used were polyamide (eight patients), polysulfone (two), or hemophane (five). All patients received erythropoietin with a mean dosage of 9700 ± 5200 IU/week (range 4000–24 000 IU/week); their mean haematocrit was 0.33 ± 0.04 (Table 1). None of the patients had received blood transfusions over the preceding 6 months nor had they received antioxidant drugs such as vitamin E or selenium as an essential cofactor of GSH-PX.

The erythrocyte redox state was determined in all patients and controls; the EPR measurements and the determination of GSH-regeneration rate were performed in 10 patients and 10 controls each.

Venous blood was collected in heparinized tubes—in the case of HD patients before the start of their HD sessions. The samples were centrifuged at 3000 r.p.m. for 7 min to separate RBCs and plasma. The buffy coat was removed carefully by aspiration. The RBCs were then washed twice in phosphate-buffered saline (PBS). During these procedures, we kept the temperatures of the samples either at 37°C, for the investigation of the antioxidant capacity with EPR and GSH regeneration rate, or at 4°C for determination of the redox state.

**Reagents**

PBS Dulbecco without Ca and Mg was obtained from Biochrom KG (Berlin, Germany). Fifteen millimolar N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES)—for maintaining a constant pH during the in vitro experiments—and 3.5 mM glucose were added to the PBS, the pH was adjusted to 7.4. All reagents were obtained from Sigma (Deisenhofen, Germany) except 5,5-dimethylpyrroline-N-oxide (DMPO) was purchased from Fluka (Neu-Ulm, Germany).

**t-BOOH treatment**

The washed and packed erythrocytes of 10 HD patients and 10 controls were mixed with the modified PBS at a ratio of 1:2 and then shaken gently in a water bath at 37°C until t-BOOH was added. Separately, 3 ml of the purified packed RBCs were incubated at 37°C for 10 min in 9 ml of the PBS (1:3) containing 10 mM N-ethylmaleimide (NEM) for GSH trapping. Thereafter, the RBCs were washed, centrifuged,
and mixed with PBS (1:2) in the same manner as the NEM-untreated erythrocytes. t-BOOH was added to the incubated RBCs, achieving a final concentration of t-BOOH of 0.324 mmol/l.

**EPR measurements**

The samples (250 μl) for EPR were taken at immediately before (time zero) and 1, 3, 5, 7, and 10 min after the addition of t-BOOH. The probes were rapidly vortexed with 250 μl of ice-cold 100 mM DMPO serving as a spin trap, and then frozen in liquid nitrogen until EPR measurements. The EPR spectra were recorded with an ESP 300E spectrometer (Bruker, Karlsruhe, Germany). The spectrometer’s settings were: centre field 3486 G, sweep width 100 G, microwave power 20 mW, frequency 9.7947 GHz, time constant 40.96 ms, sweep time 167.7 s, and receiver gain 5×10^5. The thawed, ice-cold samples were placed in a quartz EPR flat cell and measured immediately—the DMPO adducts in the flat cell are stable at room temperature for 10 min only, and start to decompose after this time. The relative concentrations of DMPO radical adducts were calculated by double integration with the WINEPR-software (Bruker, Karlsruhe, Germany).

**Determination of GSH regeneration rate**

Samples (500 μl) were drawn at time zero and 1, 3, 5, 7, 10, and 15 min after the addition of t-BOOH and were denaturated with 30% (w/v) trichloroacetic acid (TCA) containing 1 mM EDTA. The GSH content of the acidic supernatant was measured as the absorbance (λ = 340 nm) of the adduct S-(2,4-dinitrophenyl)glutathione formed after the conjugation of 1-chloro-2,4-dinitrobenzene with GSH catalysed by glutathione-S-transferase (GST) (according to the method of Vina et al. [16]).

**Erythrocyte redox state**

For the measurement of GSH, 500 μl of washed and packed RBCs were added to 500 μl of ice-cold 30% (w/v) TCA, and GSH was determined as described above. GSSG was determined according to the procedure of Vina et al. [16] by adding 250 μl of 12% (w/v) perchloric acid (PCA) containing 40 mM NEM and 2 mM bathophenanthroline-disulfonic acid (BPDs) to 250 μl of packed RBCs. The samples were derivatized with 1% 1-fluoro-2,4-dinitrobenzene (FDNB) after centrifuged and neutralized with 2 M potassium hydroxide (KOH) containing 0.3 M 3-(N-morpholino)propanesulfonic acid (MOPS). For HPLC-analysis, we used an NH2-Spherimage 80 column (5 μm, 250 x 4 mm, Knauer, Berlin, Germany). Solvent B and the binary gradient programme of Vina et al. [16] were slightly modified. Solvent A contained 80% methanol, solvent B contained 0.3 M sodium acetate in 43% methanol. The flow rate was 0.6 ml/min. The mobile phase was held at 80% solvent A in the first 5 min after sample injection followed by a linear gradient from 20% to 99% solvent B for the next 7 min. The mobile phase was then held at 99% solvent B until elution of GSSG.

The pyridine coenzymes (NAD\(^+\), NADH, NADP\(^+\), NADPH) were determined using a reverse HPLC method described earlier by our group [17]. To prevent a breakdown of the coenzymes during preparation we used an acid denaturation of the packed RBCs for the determination of the oxidized pyridine coenzymes and an alkaline denaturation for the determination of the reduced pyridine coenzymes. HPLC-analysis was performed with a C18-Eurospacer 100 column (5 μm, 250 x 4 mm; Knauer, Berlin, Germany) using a binary gradient programme. Solvent A contained 0.2 M K\(_2\)HPO\(_4\)·KH\(_2\)PO\(_4\), solvent B contained 0.2 M K\(_2\)HPO\(_4\)·KH\(_2\)PO\(_4\) with 20% (v/v) methanol. The pH was adjusted to pH 6.4 for oxidized pyridine coenzymes and to pH 7.6 for reduced pyridine coenzymes.

**Statistics**

The statistical analysis of the results was done using the two-tailed Mann–Whitney test. P < 0.05 was considered significant. The calculation of activity in GSH regeneration was done by linear regression analysis.

**Results**

The EPR spectra following treatment of the RBCs by t-BOOH can be depicted as a composite of two different radical species, the DMPO/tert-butoxy-radical adduct (DMPO/tBuO\(^-\)) and the DMPO methyl radical adduct (DMPO/CH\(_3\)) as shown in Figure 1 [13]. The EPR spectrum is characterized by the EPR spectral parameters g = 2.0054, a\(_N\) = 15.0 G, a\(_H\) = 16.2 G for DMPO/tBuO\(^-\) and g = 2.0053, a\(_N\) = 15.9 G, a\(_H\) = 22.5 G for DMPO/CH\(_3\).

The time course of the EPR signal intensity of the generated DMPO radical adducts in the erythrocytes of HD patients and controls without GSH trapping is shown in Figure 2. There was no difference in the EPR signal intensity between patients and controls from 0.5 to 2 min after t-BOOH treatment (Figure 2). The resonance signals disappeared completely in the following 2 min.

When the RBCs were pre-treated with NEM to inhibit the GSH system, we observed in both groups

![Image](Fig. 1. EPR spectrum of DMPO adducts of free radicals formed by reaction of t-BOOH with erythrocytes at 37°C. (A) experimental spectrum, (B) simulation of spectrum kA consisting of a superposition of DMPO/tBuO\(^-\) (g = 2.0054, a\(_N\) = 5.0 G, a\(_H\) = 16.2 G, peak to peak linewidth = 0.9 G), and DMPO/CH\(_3\) (g = 2.0053, a\(_N\) = 15.9 G, a\(_H\) = 22.5 G, peak to peak linewidth 0.9 G).)
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Fig. 2. Time-course of concentration (mean ± SD) of DMPO radical adducts in erythrocytes of HD patients (n = 10) and controls (n = 10) treated with 0.324 mM t-BOOH with and without inhibition of GSH system by NEM treatment (*P = 0.005, **P = 0.001).

Fig. 3. Kinetic profile of GSH recovery in RBCs of HD patients (n = 10) and controls (n = 10) treated with t-BOOH at 37°C. The calculated time rate in GSH regeneration is for: HD patients (—) 99.5 ± 13.5 nmol/min×ml RBCs; controls (—) 94.2 ± 16.9 nmol/min×ml RBCs. *P = 0.001.

Discussion

The antioxidant capacity of erythrocytes usually is determined by the measurement of various antioxidant enzymes, along with their substrates, and of other antioxidants such as vitamins C and E. We, however, used EPR for a direct determination of total antioxidant capacity in erythrocytes after inducing an artificial oxidant stress in vitro. Even after a strong oxidant stress as with 972 nmol of t-BOOH/ml RBCs, only a low level of the DMPO radical adducts was measured in RBCs—indicating a strong erythrocyte antioxidant capacity for hydroperoxides both in controls and in patients. These findings support the hypothesis that the correction of anaemia followed by an increase in the blood level of GSH improves the total blood antioxidant power [18].

The simultaneous decrease of GSH confirms that the hydroperoxide t-BOOH is eliminated to a large extent by enzymatic GSH oxidation. This GSH oxidation primarily is catalysed by the cytosolic GSH-PX, but GST may also play an role in the detoxification of organic hydroperoxides—because GST shows a GSH-PX-like activity and the GST activity is highly overexpressed in uraemic patients [19]. GSH also is used by the phospholipid hydroperoxide GSH-PX, an enzyme which is able to reduce esterified fatty acid and cholesterol hydroperoxides. After GSH-blocking with NEM, we found: (i) a sharp increase in radical concentration, indicating the important role the GSH system plays in eliminating hydroperoxides and (ii) a significant delay in the elimination of free radicals in the RBCs of HD patients compared with the controls, pointing to a decrease in antioxidant capacity outside the GSH system. By trapping GSH with NEM, t-BOOH can no longer be scavenged by the GSH system. The subsequent decomposition of t-BOOH facilitated by Fe2+ leads to a generation of peroxyl and alcoxyl radicals. These radical species are eliminated only by non-enzymatic antioxidants—either by the liposoluble scavengers vitamin E or ubiquinol or by the hydrosoluble antioxidants β-carotene, protein thiols or bilirubin [1]. In RBCs, vitamin E plays
the most important role among these substances, especially as an inhibitor of the free-radical chain reaction of lipid peroxidation [1]. A decrease in the lipophilic, membrane-protecting vitamin E is thought to cause an increase of oxidative damage to the cell membrane. A decrease of RBC vitamin E in HD patients, despite normal serum vitamin E levels, was observed by Cristol et al. [3]. These authors also showed that vitamin E supplementation can reduce the required dose of erythropoietin, and have suggested that oxidative stress could be one of the reasons for resistance to erythropoietin in HD patients. The decreased capacity for free-radical scavenging after GSH blocking in our HD patients, accounts for the impairment of antioxidant forces outside the GSH system.

An impaired HMPS in uraemic patients was first suggested by Yawata et al. [10]. The elevated GSSG and the decreased NADPH levels measured by Canestrari et al. in the RBCs of HD patients would support this theory [8]. Costagliola et al. did not, however, find an increased GSSG level in the RBCs of HD patients [6]. Chu et al. described an unaffected HMPS activity measured by liberation of $^{14}$CO$_2$ from $^{14}$C-labelled glucose in incubated erythrocytes of HD and CAPD patients [9]. Because of these conflicting results, we decided to determine the activity of GSH regeneration in RBCs after the addition of tert-BOOH, without any difference between HD patients and controls [8,10]. The slower elimination of peroxyl and alcoxyl radicals, generated by tert-BOOH after inhibition of the GSH system in HD patients compared with controls indicates a defect in the antioxidants outside the GSH system and could be one reason for the reduced life span of erythrocytes in the uraemic state. Our results also point to the benefits of correcting anaemia for lowering oxidative stress; erythrocytes are powerful antioxidant scavengers, and an increase in erythrocyte number increases the antioxidant potential of blood.

Furthermore, all patients in our study received erythropoietin to maintain a haematocrit above 30%. Erythropoietin treatment is not mentioned in the study of Canestrari et al. and can be excluded in the report of Yawata et al. [8,10]. It is known that RBCs will lose their enzyme activities with ageing. The treatment of uraemic patients with erythropoietin leads to an increased number of young RBCs with higher enzyme activities and elevated ATP [21].

Because our results are obtained in vitro, we cannot exclude the possibility that the presence of substances in the uraemic plasma can modify the GSH-related detoxification in vivo. Galli et al. described an inhibition of RBC-GST activity with uraemic plasma; Canestrari et al. reported an increase of plasma-GSH-PX activity after HD, but this effect was not observed in the case of RBC-GSH-PX and RBC-GSSG-Rd [8,19]. Although we did not observe a deficiency in the RBC-GSH system of HD patients, it is important to note that our findings in RBCs are not in conflict with the known deficiency in the plasma GSH system of uraemic patients shown by Ceballos-Picot et al. [7].

In conclusion, by using EPR we could show that the antioxidant potential of erythrocytes is extremely high for the hydroperoxide tert-BOOH, without any difference between HD patients and controls. The slower elimination of peroxyl and alcoxyl radicals, generated by tert-BOOH after inhibition of the GSH system in HD patients compared with controls indicates a defect in the antioxidants outside the GSH system and could be one reason for the reduced life span of erythrocytes in the uraemic state. Our results also point to the benefits of correcting anaemia for lowering oxidative stress; erythrocytes are powerful antioxidant scavengers, and an increase in erythrocyte number increases the antioxidant potential of blood.

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References


Table 2. RBC reduced and oxidized glutathione and reduced and oxidized pyridine coenzymes (mean±SD) in HD patients (measured before HD) and controls (*P = 0.00001)

<table>
<thead>
<tr>
<th></th>
<th>GSH (nmol/ml RBCs)</th>
<th>GSSG (nmol/ml RBCs)</th>
<th>GSH/GSSG ratio</th>
<th>NADPH (nmol/ml RBCs)</th>
<th>NADP (nmol/ml RBCs)</th>
<th>NADH (nmol/ml RBCs)</th>
<th>NAD (nmol/ml RBCs)</th>
</tr>
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<tbody>
<tr>
<td>HD patients (n = 15)</td>
<td>1858 ± 439</td>
<td>47.6 ± 14.4</td>
<td>44.5 ± 21.7</td>
<td>15.0 ± 3.2</td>
<td>50.4 ± 6.0</td>
<td>3.2 ± 0.6</td>
<td>74.2 ± 9.5*</td>
</tr>
<tr>
<td>Controls (n = 15)</td>
<td>1831 ± 423</td>
<td>45.8 ± 17.5</td>
<td>45.1 ± 18.2</td>
<td>16.8 ± 4.8</td>
<td>50.2 ± 9.5</td>
<td>3.3 ± 0.9</td>
<td>55.8 ± 9.4*</td>
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**Editor’s note**

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