# Effect of hemodialysis on the antioxidative properties of serum

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## Abstract

In patients with chronic renal failure undergoing regular hemodialysis (HD), oxidative stress is involved in the development of dialysis-related pathologies. The aim of the study was to measure the effect of HD treatment on the general antioxidative status of serum with special consideration of the specific oxidizability of lipids and proteins.

Indicators for the oxidative/antioxidative status of plasma were monitored at the beginning and at the end of a dialysis session on the arterial and venous side of the dialyzer. A decrease in the antioxidant status was accompanied by an increased oxidizability of proteins as well as lipids during HD treatment. During the first passage of the dialyzer, the lag time of lipid oxidation decreased from $114.0 \pm 19.8$ to $81.5 \pm 18.9$ min, the lag time of protein oxidation decreased from $105.0 \pm 24.6$ to $72.9 \pm 21.3$ min and the total antioxidative status decreased from $518 \pm 24$ to $252 \pm 124$ μM trolox equivalents. The carbonyl content of serum proteins was high in patients with end stage renal disease (ESRD) ($3.9 \pm 1.1$ vs. $0.9 \pm 0.1$ nmol/mg in controls) but did not change significantly during dialysis procedure.

Our data demonstrate that the susceptibility of serum lipids and proteins to oxidative modification is severely increased by HD treatment.
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*Keywords:* Hemodialysis; End stage renal disease; Antioxidant; Free radical; Oxidative stress

## 1. Introduction

There is wide agreement that patients undergoing regular dialysis treatment experience increased oxidative stress [1,2]. The lines of evidence range from increased stimulation of neutrophiles [3,4], elevated serum concentrations of lipid peroxidation products [5,6], impairment of the antioxidant system [7–9], endothelial dysfunction [9], to increased modification of proteins [10]. However, the sources and mechanisms of elevated oxidative damage remain a matter of controversy. Some authors provide evidence that dialysis treatment is the main source of increased oxidative damage in patients with end stage renal disease (ESRD) [7,9,11]. Others discuss the disease itself as the main reason for elevated oxidative stress [12,13]. The methods used for the determination of oxidative stress are generally of a broad range. An often used and easily detectable parameter for the

serum antioxidative properties is the total antioxidant status (TAS) [14]. However, this value is predominately caused by the uric acid and protein content of serum [15]. As these values are disturbed in ESRD patients, TAS has to be interpreted with care especially concerning ESRD patients [16–18]. On the other hand, as uric acid and protein contribute to the antioxidative property of serum, TAS could be a valuable tool to follow changes during the dialysis treatment. Moreover, there are conflicting results concerning the antioxidative status of serum from ESRD patients. Impairment of the antioxidant system as well as an increased antioxidative status were reported, obviously depending also on the method used [7,8,17–22].

The oxidizability of serum components provides an easily accessible parameter for determining antioxidative properties of serum. However, measuring oxidation of isolated serum components may lead to an underestimation of antioxidative capacities due to the loss of antioxidants during preparation. Therefore, the measurement of oxidizability of serum components in the whole serum is a convenient and reliable alternative allowing a measurement in a less artificial environment [23,24]. Further problems

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involved in all the methods are the questions which prooxidant to use and, on the other hand, the oxidizability of the target molecule [24].

This study was intended to follow the antioxidative properties of serum samples from ESRD patients during the hemodialysis (HD) procedure on the basis of different methods including TAS, the lag times of oxidation of protein and lipid in serum as well as the carbonyl content of serum proteins.

## 2. Materials and methods

### 2.1. Patients

Twenty-two patients (13 males, 9 females, mean age 69.7 years, range 40–90 years) with ESRD who have undergone chronic HD treatment three times a week for 37.4 months (range 4–96 months) were included in this study after having given their informed consent. Blood samples were drawn from the arterial and venous side of the dialyzer 5 min after the beginning and 5 min before the end of the dialysis session. Samples were collected using vacuum tubes, and aliquots of isolated serum were stored at $-70$ °C.

The etiologies of the patients renal diseases were diabetic nephropathy (4), primary glomerulonephritis (5), polycystic disease (2), plasmocytoma (2), nephrosclerosis (4) and unknown (5). One patient with nephrosclerosis had a heart transplantation in 1988, one patient with glomerulonephritis had a kidney transplantation in 1996. Suffering from chronic rejection, he came back to dialysis 4 years later. Both patients are given immunosuppressive therapy.

### 2.2. Dialysis treatment

During the study, patients were kept on their regular dialysis regimen. Using Gambro AK 200 machines 16 patients had 4 h HD treatment. The synthetic filters used were Lundia pro 600® ($n = 3$), Lundia pro 800® ($n = 3$), both gambrane membrane, GFS plus16® ($n = 6$), a hemophane membrane (Gambro, Lund, Sweden), and F6HPS® ($n = 4$), a polysulfone membrane (Fresenius, Bad Homburg, Germany). Blood flow was 300 ml/min, dialysate flow 500 ml/min. In six patients, hemodiafiltration was performed for 4.5 h with either Polyflux 8 L® ($n = 3$), a polyamide filter (Gambro), or Tricea 210 G® ($n = 3$) a triacetate membrane (Baxter, Vienna, Austria), with a blood flow rate of 300 ml/min, and a dialysate flow of 700 ml/min. Blood was drawn immediately before the inlet of the dialyzer cartridge and immediately at the outlet of the cartridge. Serum was prepared and frozen at $-70$ °C until analysed.

### 2.3. Antioxidant status

A commercial test kit, Randox TAS, (Dr. Franz Tatzber KEG, Klosterneuburg, Austria) was used. The assay is based on the formation of the radical cation of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate], (ABTS$^+$) and its reduction by antioxidants [14,25]. The solutions of the test kit were prepared according to the instructions and contained the following constituents: chromogen, consisting of 6.1 μM metmyoglobin and 610 μM ABTS; substrate, 250 μM hydrogen peroxide as well as a standard of 1.6 mM trolox. The assay was not performed as a single time point but as a kinetic measurement of the lag time of the onset of radical formation [14,25]. For the assay, 10 μl of sample were mixed with 250 μl chromogen solution in a cavity of a 96-well plate. Substrate (50 μl) was added to the mixture and the measurement of the absorbance at 620 nm was started immediately. From the kinetic curves of blank (water) and standard the start time of the reaction was calculated using the MS Excel© software. In this procedure, slightly different handling times of different plates are taken into account. The lag times of the samples were calculated and compared to the values obtained for trolox. The antioxidant status of the samples was then expressed as micromolar trolox equivalents. Measurements were performed in duplicate.

### 2.4. Fluorescence oxidation assay

The measurements were performed as described elsewhere [24]. An ethanolic solution (5 μl) of fluorophore, 1,6-diphenylhexatriene propionic acid (DPHPA) for protein labelling and 1-palmitoyl-2-((2-(4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl)ethyl)-carbonyl-*sn*-glycero-3-phosphocholine (DPHPC) for lipid labelling was added to 150 μl of an argon-saturated serum sample. For labelling with DPHPC, the reaction mixture was kept under argon at 37 °C for 12 h, whereas for DPHPA-labelling, the mixture was incubated under argon at room temperature for 1 h. A 50-μl aliquot of the labelled sample was diluted with 150 μl of air-saturated PBS-buffer. Oxidation was started by adding 100 μl of 90 mM 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH, 30 mmol/l final concentration) dissolved in phosphate buffered saline (PBS) and monitored by following the time-dependent decrease of the fluorescence intensity at 430 nm (excitation at 354 nm) at 37 °C using a fluorescence well-plate reader Perkin-Elmer LS50B and 96-multiwell plates from Perkin-Elmer (Norwalk, USA). Measurements were performed in triplicate. Lag times for the DPHPA oxidation are denoted as protein lag times, and those for DPHPC are denoted as lipid lag times.

### 2.5. Protein carbonyl content

The carbonyl content of proteins was analysed according to Buss et al. [26] with slight modifications. Oxidized bovine serum albumin (BSA) was prepared for standardisation as described elsewhere [29]. Serum samples and standards were diluted to give a final protein concentration of 4 mg/ml. The protein derivatization was carried out in 1-ml tubes with 45 μl of DNPH solution (10 mM dinitrophenylhydrazine; in 6

Table 1
Clinical parameters of the patients before dialysis

| Patients (n) | 22 | Normal range |
|---|---|---|
| Sex | 13 males/9 females | |
| Age (a) | $69.7 \pm 13.2$ | |
| Total proteins (g/100 ml) | $6.5 \pm 0.8$ | 6.6–8.3 |
| Albumin (g/100 ml) | $3.2 \pm 0.5$ | 3.5–5.3 |
| Creatinine (mg/100 ml) | $6.6 \pm 1.9$ | 0.6–1.3 |
| Urea (mg/100ml) | $120 \pm 40$ | 10–45 |
| Uric acid (mg/100 ml) | $6.6 \pm 1.1$ | 2.4–5.7 |
| Total cholesterol (mg/100 ml) | $180 \pm 60$ | <200 |
| Triglycerides (mg/100 ml) | $170 \pm 50$ | <150 |
| Iron (µg/100 ml) | $37 \pm 19$ | 50–160 |
| Transferrin (mg/l) | $1458 \pm 467$ | 2000–3600 |

M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5) added to 15 µl of serum samples or standards. The mixtures were incubated at 25 °C for 45 min, being vortexed every 10 min. Then 50 µl of each sample were added to 950 µl of coating buffer (10 mM PBS, pH 7.4). Duplicate 100-µl aliquots were added to wells of a white 96-well chemiluminescence microtiter plate (Greiner, Kremsmünster, Austria) and incubated overnight at 4 °C. After washing three times with PBS (PAA, Linz, Austria), 100 µl of a 1:10,000 diluted anti-DNP–HRP in 10 mM PBS, pH 7.4 were added to each well and incubated for 1 h at 25 °C. After washing five times with PBS, the chemiluminescence was measured on a chemiluminescence reader (Lumistar, BMG, Germany) after adding 100 µl/well Super Signal Maximum Sensitivity substrate (Pierce, Rockford, USA).

### 2.6. Statistics

The indicated data are means $\pm$ S.D. Data were compared using a paired $t$-test. The significance of correlation is based on linear regression.

## 3. Results

Pre-dialysis serum samples were tested for their clinical characteristics. Table 1 shows these parameters. The total protein and albumin content, as well as iron and transferrin concentrations are slightly below the normal range. Creatinine and urea are dramatically increased, as expected. Lipid and uric acid contents are partly above the recommended range.
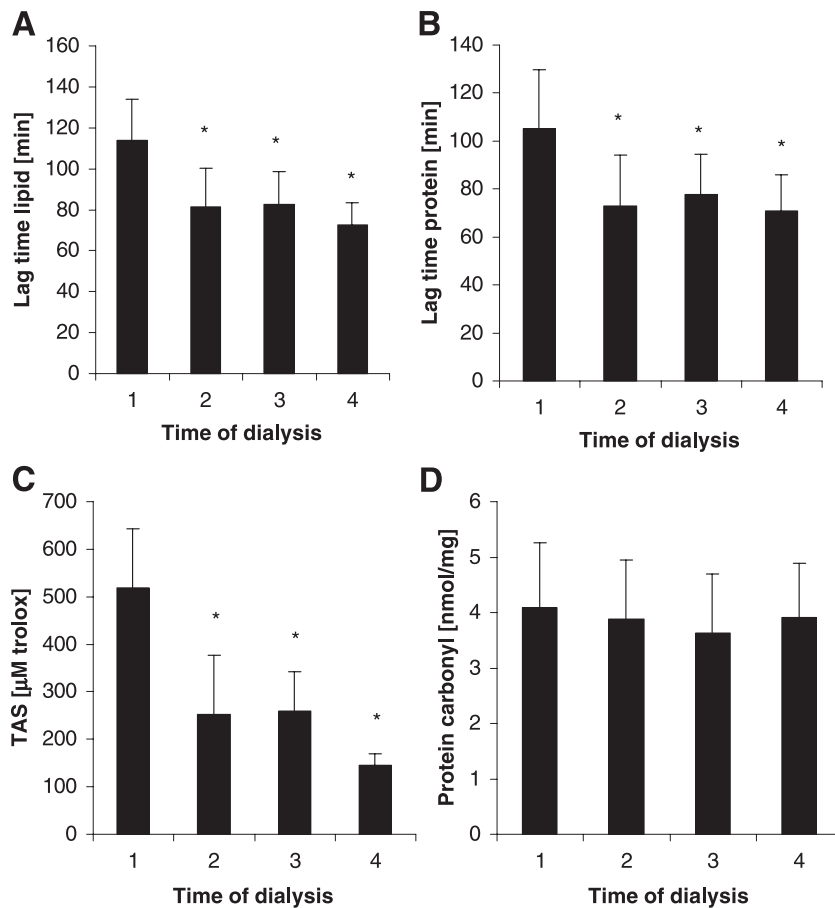


Fig. 1. Antioxidative and oxidation parameters of serum during hemodialysis. Blood samples were collected at the beginning (1 and 2) and at the end (3 and 4) of the HD session, at the inlet (1 and 3) or at the outlet (2 and 4) of the dialyzer. The lag times of lipid (A) or protein (B) oxidation, the total antioxidative status (C) and the carbonyl content of proteins in serum were determined. Bars indicated with ∗ are significantly different from the initial value ($P < 0.001$).

The dialysis treatment is accompanied by an increase in the susceptibility of serum components to damage by free radicals. As shown in Fig. 1A, the lag time of oxidation of serum lipids has already significantly decreased during the first dialyzer passage ($114.0 \pm 19.8$ vs. $81.5 \pm 18.9$ min, $P < 0.001$). This value is held almost constant during further dialysis and becomes slightly lower at the end of the treatment. Compared to a group of healthy controls analysed during the same study ($n = 9$), the lag time of lipid perox-idation in ESRD patients before HD treatment is not significantly different (controls $118 \pm 10$ min, $P = 0.737$). In a similar fashion, the lag times of protein oxidation have decreased during the dialysis treatment ($105.0 \pm 24.6$ vs. $72.9 \pm 21.3$ min) before and after the first dialyzer passage ($P < 0.001$), as is shown in Fig. 1B. Again ESRD patients are in the control range ($110 \pm 7$ min, $P = 0.999$) at the beginning of the dialysis session. When measuring the TAS, we found out that ESRD patients have values which are in the range of a control group ($518 \pm 124$ vs. $505 \pm 82$ μM trolox in control). However, as shown in Fig. 1C, the TAS has dramatically decreased during the HD treatment. After the first dialyzer passage, only about 50% remain. The serum TAS has even further decreased during the last passage through the dialyzer (from $250 \pm 83$ to $145 \pm 24$, $P < 0.001$) to a value representing only about 28% of the initial value.

For both lag times, protein and lipid, good correlations were found with the TAS values ($r = 0.901$, $P < 0.001$ for the lipid lag time and $r = 0.839$, $P < 0.001$ for the protein lag time) as shown in Fig. 2. The TAS values measured at the start of the HD treatment correlated well with the urate content ($r = 0.816$, $P < 0.001$) and no significant correlation of TAS was found with albumin, protein or any other clinical parameter measured.

The carbonyl content of serum proteins of patients with ESRD was found to be about fourfold compared to a group of healthy controls ($3.9 \pm 1.1$ vs. $0.9 \pm 0.1$ nmol/mg,
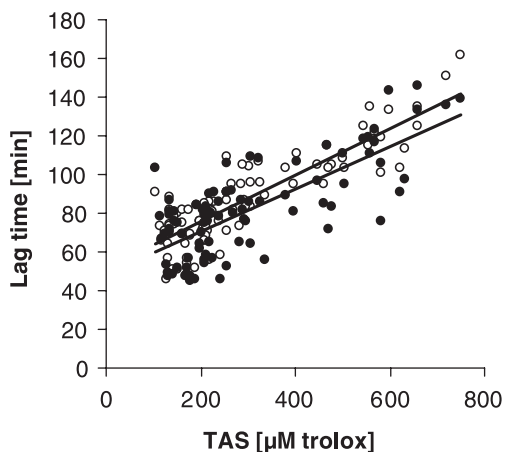
$P < 0.001$). Throughout the dialysis procedure this value did not change significantly.

## 4. Discussion

The antioxidative status of body fluids is a valuable parameter to assess the sum of antioxidants in these systems including their synergistic effects. However, the interpreta-tion of data of the antioxidative status has to be done with care [16]. Especially concerning the situation of ESRD patients, the value of the TAS has been questioned and the results already available are rather controversial. The differences reported are at least partly due to the differences in the methods used. Selecting oxidation probes for the assessment of antioxidative status, one has to take care regarding the oxidation susceptibility of the probe. As we have previously shown, the fluorescence probes used in our study have oxidation susceptibilities very similar to natural polyunsaturated fatty acids and albumin, respectively [24]. Therefore, these probes can be used as markers for the oxidation of lipoproteins and aqueous phase components of human serum.

Using the ABTS-based method, we found no significant differences in TAS between controls and ESRD patients. Patients before the HD treatment showed elevated levels of serum urate but albumin contents below the normal range. This may explain the lack of a net effect in the TAS which is predominated by albumin and urate. In parallel, the oxidiz-ability of water- and lipid-soluble serum components of controls and patients are in the same range. The dramatic decrease of the TAS during the HD session is in agreement with the parallel decrease of the lag time of the protein oxidation. This could be easily explained by a loss of ascorbic acid and urate during HD [19,27,28,32], which synergistically act as antioxidants. Surprisingly, the oxidiz-ability of lipid-soluble components also increased during the HD treatment. Published data concerning vitamin E contents in serum of HD patients range from no difference compared to controls [5,20,29–32] to lowered values in patients [33–35]. There is some evidence, however, that vitamin E is partly lost during the dialysis session [29,36]. Together with the lost synergistic effect of ascorbic acid, this explains the decrease in lag times of lipid oxidation.

Our data resulting from different methods are in agree-ment with the published works reporting an impairment of the serum antioxidant system during HD treatment [7–9]. The oxidation sensitivity of water-soluble components is increased during the HD session. On the other hand, carbonyl content of proteins as a long-term parameter of oxidative damage is not changed during HD treatment. However, the content of carbonyl groups is severely in-creased in patients compared to controls. Therefore, HD treatment means a contribution to sensitize serum compo-nents against oxidative stress but the damage itself does not only happen during HD sessions.



Fig. 2. Correlation of oxidation lag times of lipids (○) and proteins (●) with TAS values.

The good agreement of the different methods applied, using different sources of radicals as initiators shows that measurement of TAS is valuable when following this parameter within a group of persons. However, comparing different groups like HD patients with control subjects may lead to misinterpretations as the increased level of protein carbonyl groups does not match the normal value of TAS and lag times before HD.

We have shown that the loss of antioxidants is accompanied by an increase in oxidizability of proteins as well as lipids using oxidation markers which have the same oxidizability as the natural targets of oxidation [24]. The pronounced decrease in TAS at the beginning of HD treatment might be due to a response to both a loss of urate and ascorbic acid, and a particular high oxidative stress during this first dialyzer passage.

At the end of the session, a further decrease in TAS might be predominated by a further loss of urate and ascorbic acid. On the other hand, lag times of lipid and protein oxidation are decreased only at the beginning of the HD treatment responding to the antioxidant loss in a different manner or as a consequence of a massive oxidative stress during the first few dialyzer passages. It has recently been reported that free radical production is highest during the first hour of HD treatment [28]. As a reason for such a first pass effect of oxidative damage during HD treatment the bioincompatibility of the dialyzer membranes and the resulting effects on leukocyte and platelet activation have been discussed [36–39]. A lack of change of protein carbonyl groups during HD treatment combined with a rather high value of carbonyl content could be interpreted in a delay of carbonyl formation or in other sources of oxidative stress besides HD treatment in ESRD patients. The high value of uric acid content in the serum of HD patients may contribute to their normal value in TAS and oxidation lag time assay [40]. However, this normal antioxidative behaviour is not sufficient to maintain normal values of protein carbonyl groups.

Our data support the view that supplementation with antioxidants should be of some benefit for ESRD patients [41] and the necessity for such a supplementation is highest at the beginning of the HD session. Vitamin supplementation is not unusual for HD patients. Vitamin E and ascorbic acid are the most important antioxidant vitamins used [35,37,42]. Ascorbic acid, however, is primarily applied for the improvement of the iron status of HD patients and the effects on oxidative stress are often not measured [43–45]. The usefulness of the supplementation of HD patients with ascorbic acid for the improvement of the antioxidant defence has even been questioned [46,47]. Vitamin E has successfully been used to prevent oxidative damage in ESRD patients [41]. Especially oxidative stress caused by the treatment of iron deficiency, which could be prevented by supplementation with vitamin E [30]. Besides oral supplementation, vitamin E has been used for coating the dialyzer membranes and this seems to be the most efficient application [9,27,37,48]. The reason might be that oxidative stress is then suppressed where it originates and that vitamin E is not only an antioxidant but also enhances the biocompatibility of the membranes by its contribution to control leukocyte activation [37,49].

## Acknowledgements

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