

Original Article

Myeloperoxidase serves as a marker of oxidative stress during single haemodialysis session using two different biocompatible dialysis membranes

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Abstract

Background. There is increased oxidative stress in patients undergoing haemodialysis (HD); however, little is known of how different dialysis membranes contribute to the oxidative stress induced by the dialysis procedure *per se*. We therefore studied the influence of two different dialysis membranes on oxidative stress during HD.

Methods. Eight patients undergoing HD three times per week were enrolled in this cross-controlled study. Patients sequentially received HD using polysulphone (PS) and regenerated cellulose (RC) dialysis membranes for 1 week each. Blood samples were collected in the last section of each hollow fibre 0, 15, 120 and 240 min after starting HD. We determined superoxide anion production derived from neutrophils, superoxide dismutase (SOD) and glutathione peroxidase (GPx) derived from washed red cells, plasma myeloperoxidase (MPO), plasma thiobarbituric acid-reactive substances (TBARS), plasma advanced oxidation protein products (AOPP) and serum 8-hydroxy-2'-deoxyguanosine (8-OHdG).

Results. Leukocyte numbers, including neutrophils, lymphocytes and monocytes, decreased significantly after 15 min of dialysis, more so with RC than with PS membrane. For both membranes, superoxide anion production transiently increased during the first 15 min whereas the post-dialysis production was decreased. Plasma MPO levels persistently increased during dialysis with the two membranes. Moreover, the increase was more marked with RC than with PS membrane. AOPP and 8-OHdG levels increased progressively

when using RC membranes. There were no significant differences in SOD, GPx, TBARS, AOPP and 8-OHdG levels between the two membranes.

Conclusions. The biocompatibility of the dialyser affects oxidative stress production during a single dialysis session. The measurement of MPO may serve as a reliable marker of the degree of oxidative stress induced using dialysis membranes of different biocompatibilities.

Keywords: antioxidants; biocompatibility; haemodialysis; myeloperoxidase; oxidative stress

Introduction

End-stage renal disease (ESRD) patients have a high prevalence of associated disease and low survival rates and quality of life [1]. Recently, it has been proposed that exposure to an increased oxidative burden related to inflammation and diseases such as immunodeficiency and cardiovascular disease plays an important role in the pathogenesis of the associated diseases, particularly in haemodialysis (HD) patients [2].

Oxidative stress is defined as a loss of the normal balance between reactive oxygen species (ROS) production and the antioxidant system [3]. Indeed, in addition to an excess generation of ROS, uraemic patients have a decreased antioxidant capacity, which causes oxidative damage to cells. Superoxide anion (O_2^-) is a major ROS and can be converted to hydrogen peroxide, which can be used by activated myeloperoxidase (MPO) to produce the strong oxidant hypochlorite [4]. MPO or hypochlorite may further mediate oxidative modification of lipids, proteins and DNA, an effect that has been shown to be increased in HD patients [5–7].

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Conversely, superoxide dismutase (SOD), catalase and the glutathione system provide most of the protection against damage by oxygen radicals.

The causes of oxidant stress in HD patients are poorly understood. Uraemic toxins, dialyser interactions and dialysate contaminants have been suggested as the three major causes of oxidative stress [8]. Excessive generation of ROS resulting from activation of peripheral blood cells interacting with the dialyser membranes is proposed to be an important contributor. The dialysis membranes used in HD seem to play a central role in the increased production of oxygen free radicals in ESRD patients [9].

An imbalance between oxidants and antioxidants has been suggested in uraemic patients on HD. However, the respective net oxidative imbalance due to the dialysis procedure *per se* is still a matter of debate. To compare the effects of regenerated cellulose (RC) membranes and synthetic polysulphone (PS) membranes on the production of oxidative stress and antioxidants during HD, we determined superoxide anion (O_2^-), MPO, SOD, glutathione peroxidase (GPx), thiobarbituric acid-reactive substances (TBARS), advanced oxidation protein products (AOPP) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) production during dialysis.

Subjects and methods

Patients

The study protocol was approved by the Ethics Committee on Human Studies at Tri-Service General Hospital, Taipei, Taiwan. Informed consent was obtained from each patient. Eight HD patients, four men and four women, aged 40–80 years (mean: 60 ± 14 years), with a history of ESRD without other relevant systemic pathologies, such as infection, diabetes, liver disorder, malignancy or collagen-vascular disease, were enrolled in this study. The mean dialysis age was 19.5 ± 9.1 months (range: 7–33 months). All patients underwent HD three times a week for 4 h per session. The dose of dialysis was individually adjusted to maintain a $Kt/V > 1.2$. The patients' haematocrits were all > 33 –36%. Bicarbonate dialysate solutions were used: the blood flow rate was 300 ml/min and the dialysate flow rate was 500 ml/min. The colony count of micro-organisms in the water used to prepare the dialysis fluid was ≤ 200 colonies/ml. The endotoxin levels detected weekly in the dialysates were < 0.01 EU/ml. No study subjects smoked and patients taking lipid-lowering drugs, steroids, cyclosporin, erythropoietin or antioxidant supplements, such as vitamins C and E, were excluded from the study. Medications, when needed, included antihypertensive treatment, vitamin D, vitamin B complex, phosphate binders and potassium-exchanging resins.

At the time of the study, the patients had been on HD using PS dialysers (F80; Fresenius, Bad Homburg, Germany) for ≥ 6 months. During the random, cross-controlled study, each patient was studied twice: once with PS membranes (F80; Fresenius, Bad Homburg, Germany) and once with RC membranes (DC190; Dynamic Technology Corp., Hsinchu, Taiwan), for 1 week with each.

Samples

After obtaining informed consent, blood samples (10 ml) were drawn from the venous end of the arteriovenous fistula, collected into ethylenediaminetetraacetic acid (EDTA) or citrated tubes, immediately chilled in an ice-water mixture and centrifuged (1000g for 15 min at 4°C) within 30 min. Blood samples were collected at 0, 15, 120 and 240 min time-points in the HD session. The granulocytes produce the most superoxide anions and the erythrocytes are equipped with a highly effective antioxidant defence system. We determined the superoxide anion derived from neutrophils by flow cytometry and immediately measured SOD and GPx in washed red cells obtained from whole blood anticoagulated with EDTA by spectrophotometry. Plasma was removed for assay of MPO, TBARS, AOPP and 8-OHdG.

Blood cell counts

Haematological parameters, including total leukocyte numbers and differential counts of neutrophils, lymphocytes, monocytes, eosinophils and basophils, were measured using an SE-900 CBC autoanalyser (Sysmex Corp., Katogawa, Japan).

Oxidant markers

Superoxide anion assay. Non-fluorescent hydroethidine can be oxidized within cells to the fluorescent product ethidium bromide by superoxide anions produced by the cells. Neutrophil superoxide anion production was determined by measuring cell fluorescence intensity using a flow cytometry method, as described previously [10]. Briefly, leukocytes were harvested from citrated whole blood after lysis of red blood cells using an ammonium chloride lysis solution (BD Pharmingen). After centrifugation (10 min at 300g, 4°C) and washing with 2 ml phosphate-buffered saline (PBS), the leukocyte pellet was suspended in Hanks' balanced salt solution. Leukocytes were then incubated with 10 μ l dihydroethidium (Sigma, St Louis, MO, USA) for 15 min at 37°C in the dark. After labelling, leukocytes were incubated in the presence or absence of PMA (100 ng/ml; Sigma) for 15 min at 37°C. Total leukocytes were subjected to flow cytometry analysis.

Flow cytometric analysis was performed using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). Five thousand events were registered from each blood sample. The neutrophil population was determined by gating on a forwards and side-scatter dot plot and the intensity of the specific fluorescence was calculated and expressed as the relative linear median fluorescence intensity.

Myeloperoxidase. MPO analysis was performed using the Bioxytech MPO enzyme immunoassay kit (OXIS International Inc., Portland, OR, USA) according to the manufacturer's instructions. Briefly, diluted samples were pipetted into 96 wells coated with the first monoclonal antibody to MPO and incubated for 2 h at room temperature. The MPO-monoclonal antibody complex was labelled with a biotin-linked polyclonal antibody prepared from goat MPO antisera. The final step of the assay was based on a biotin-avidin coupling in which avidin has been covalently linked to alkaline phosphatase. The amount of MPO was enzymatically measured upon addition of 4-nitrophenyl-phosphate 9pNPP, by reading the microplate at 405 nm.

Antioxidant markers

Superoxide dismutase. Erythrocyte SOD activity was determined by using RANSOD kit (Randox Labs, Crumlin, UK), which is based on the method of McCord and Fridovich [11]. Xanthine and xanthine oxidase were used to generate superoxide anion radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride quantitatively to form a red formazan dye. SOD inhibits the reaction by converting the superoxide radical to oxygen. Briefly, blood was drawn in an EDTA-containing tube and centrifuged at 2000 g for 10 min at 4°C. Plasma and buffy coats were removed and the remaining erythrocytes were drawn from the bottom, washed three times with NaCl solution (0.9%) and haemolysed by the addition of an equal volume of distilled water to yield a 50% haemolysate. These haemolysates were used for the assay of SOD and GPx. Absorbance was measured at 505 nm on a Beckman DU 7500 spectrophotometer (Beckmann Instruments, Fullerton, CA, USA) for 30 s after the addition of xanthine oxidase (125 µl) as start reagent. A standard curve was prepared by using the standard provided in the kit and the value for each sample was read from this curve. The SOD activity was expressed as U/g Hb. One unit is the amount of SOD that inhibits the rate of formazan dye formation by 50%.

Glutathione peroxidase. Erythrocyte GPx activity was determined by using RANSEL kit (Randox Labs, Crumlin, UK), which is based on the method of Paglia and Valentine [12]. GPx catalyses the oxidation of glutathione by cumene hydroxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is converted immediately to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance at 340 nm was measured. The assay was performed on a haemolysate (50 µl) with 1 ml diluting agent and 1 ml Drabkin agent. A standard curve was prepared by using the standard provided in the kit and the value for each sample was read from this curve. The GPx activity was expressed as U/g Hb and the unit was defined as the enzyme activity necessary to convert 1 µmol NADPH to NADP⁺ in 1 min.

Oxidation products markers

Lipid peroxidation. Plasma TBARS were analysed by the method of Yagi [13]. Briefly, 0.5 ml of 20% trichloroacetic acid, 0.5 ml of 0.67% thiobarbituric acid:glacial acetic acid solution (1:1, v/v) and 1.5 ml of 150 mM NaCl are added to 100 ml sample. The mixture is boiled at 95°C for 1 h, TBARS are extracted with *n*-butanol and the fluorescence of the

extracted solution is analysed by spectrofluorometry (Ex: 515 nm; Em: 553 nm).

Protein oxidation. AOPP were determined using the method of Witko-Sarsat *et al.* [6]. Briefly, blood samples for determination of AOPP were collected into EDTA. The plasma was separated immediately by centrifugation and stored at -70°C until analysis. Plasma AOPP levels were assessed by spectrophotometric measurement of absorbance at 340 nm and expressed as chloramine-T equivalents after 5-fold dilution of 200 µl plasma with 20 mmol/l PBS (pH 7.4), the addition of 80 µl acetic acid and reading against a PBS blank.

DNA oxidative damage. Serum 8-OHdG levels were measured with a competitive enzyme-linked immunosorbent assay kit, using the highly sensitive monoclonal antibody N45.1 (high sensitivity 8-OHdG Check; Japan Institute for the Control of Aging, Shizuoka, Japan) [14]. Briefly, serum samples were again separated by centrifugal ultrafiltration at 10 000 g for 50 min using Microcon YM-10 (Millipore Corp., Bedford, MA, USA). Fifty microlitres of the filtration sample and an 8-OHdG monoclonal antibody were added to each well and incubated for 60 min at 37°C. After the wells were washed three times, horseradish peroxidase-conjugated antibody was added, followed by incubation for 60 min. The wells were again washed three times. Next, a substrate containing 3,3',5,5'-tetramethylbenzidine was added and the wells were incubated for 15 min. The reaction was stopped by 1 M phosphoric acid. The absorbance was read at a wavelength of 450 nm.

Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed using the Mann-Whitney test to determine the differences between groups. Wilcoxon's test was used for analysis of paired data. A *P*-value of <0.05 was considered statistically significant. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS/PC; SPSS, Inc., Chicago, IL, USA).

Results

Basal leukocytes counts, differential counts and levels of oxidants (O₂⁻ and MPO), antioxidants (SOD and GPx), lipid peroxidation (TBARS), AOPP and DNA oxidative damage (8-OHdG) were not different between the two groups at 0 min (Table 2).

Table 1. Baseline clinical and laboratory characteristics of the study population

Patient no.	Sex	Age (years)	Aetiology of ESRD	Kt/V (Gotch)	Duration of HD (months)	History of hepatitis
1	M	68	Analgesic nephropathy	1.27	29	-
2	F	68	Chronic glomerulonephritis	1.56	17	-
3	F	61	Analgesic nephropathy	1.24	7	-
4	F	40	Chronic glomerulonephritis	1.99	28	-
5	M	80	Analgesic nephropathy	1.28	8	-
6	M	45	Chronic glomerulonephritis	1.29	33	-
7	M	48	Chronic glomerulonephritis	1.54	14	-
8	F	77	Chronic glomerulonephritis	1.46	20	-

Table 2. Comparison of blood cell counts and oxidative parameters in patients undergoing haemodialysis with regenerated cellulose and polysulphone membrane

	Dialysis membrane							
	Polysulfone				Regenerated cellulose			
	0 min	15 min	120 min	240 min	0 min	15 min	120 min	240 min
Blood Cell Count ($\times 1000/\mu\text{l}$)								
Leukocytes	5.39 \pm 0.64	5.09 \pm 0.62 ⁺	5.64 \pm 0.67	5.66 \pm 0.67	5.56 \pm 0.64	1.34 \pm 0.24 ^{*#}	7.98 \pm 1.27 [#]	8.06 \pm 1.18 ^{*#}
Neutrophils	4.00 \pm 0.49	3.49 \pm 0.46 ⁺	3.93 \pm 0.49	3.82 \pm 0.48	3.45 \pm 0.39	0.31 \pm 0.08 ^{*#}	5.88 \pm 0.98 ^{*#}	6.11 \pm 0.93 ^{*#}
Lymphocytes	1.39 \pm 0.16	1.16 \pm 0.15 ⁺	1.22 \pm 0.16	1.33 \pm 0.18	1.40 \pm 0.22	0.89 \pm 0.15 ^{*#}	1.14 \pm 0.18 [#]	1.25 \pm 0.20
Monocytes	0.28 \pm 0.03	0.19 \pm 0.02 ⁺	0.22 \pm 0.02	0.26 \pm 0.02	0.33 \pm 0.03	0.05 \pm 0.01 ^{*#}	0.34 \pm 0.08	0.37 \pm 0.05 [*]
Oxidants Products								
O ₂ ⁻ (RLMFI)	10.23 \pm 0.65	11.39 \pm 0.78 ⁺	8.57 \pm 0.64 ⁺	7.29 \pm 0.56 ⁺	12.13 \pm 1.10	16.74 \pm 2.29 ^{*#}	9.39 \pm 1.02 [#]	8.09 \pm 1.01 [#]
MPO (mg/mL)	22.9 \pm 2.2	69.7 \pm 13.8 ⁺	62.7 \pm 10.9 ⁺	38.1 \pm 5.1 ⁺	22.1 \pm 2.7	139 \pm 14.2 ^{*#}	118.7 \pm 9.9 ^{*#}	103.3 \pm 20.7 ^{*#}
Antioxidant Capacity								
SOD (U/g Hb)	1110 \pm 160	1050 \pm 240	990 \pm 150	1120 \pm 130	1050 \pm 90	880 \pm 150	890 \pm 160	1080 \pm 160
GPx (U/g Hb)	44.94 \pm 13.73	62.65 \pm 15.86	37.92 \pm 15.12	38.63 \pm 12.06	46.61 \pm 8.22	40.32 \pm 9.45	51.38 \pm 9.37	58.52 \pm 9.92
Oxidation Products								
TBARS (nmol/mL)	2.52 \pm 0.33	2.39 \pm 0.37	2.51 \pm 0.48	2.84 \pm 0.56	2.47 \pm 0.29	2.49 \pm 0.44	2.03 \pm 0.16	2.70 \pm 0.30
AOPP ($\mu\text{mol/L}$)	148.1 \pm 5.0	132.0 \pm 6.4 ⁺	162.5 \pm 14.9	174.6 \pm 12.6 [*]	148.4 \pm 11.5	149.4 \pm 11.2	166.9 \pm 13.4	186.4 \pm 19.4 [#]
8-OHdG (ng/mL)	3.59 \pm 0.23	3.50 \pm 0.39	3.58 \pm 0.27	3.50 \pm 0.22	3.41 \pm 0.24	3.50 \pm 0.24 [#]	3.66 \pm 0.34 [#]	4.14 \pm 1.15 [#]

O₂⁻: neutrophil superoxide anion, SOD: erythrocyte superoxide dismutase, GPx: erythrocyte glutathione peroxidase, MPO: plasma myeloperoxidase, TBARS: plasma thiobarbituric acid-reactive substances, AOPP: plasma advanced oxidatioducts, 8-OHdG: serum 8-hydroxy-2'-deoxyguanosine (8-OHdG), RLMFI: relative linear median fluorescence intensity. **P* < 0.05 versus polysulphone. ⁺*P* < 0.05 versus polysulphone baseline values. [#]*P* < 0.05 versus regenerated cellulose baseline values.

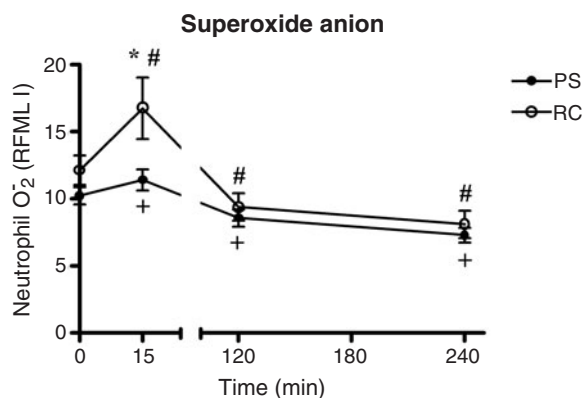


Fig. 1. Changes in superoxide anion production in patients using RC and PS membranes at various times during HD. **P* < 0.05 vs PS; ⁺*P* < 0.05 vs PS baseline values; [#]*P* < 0.05 vs RC baseline values.

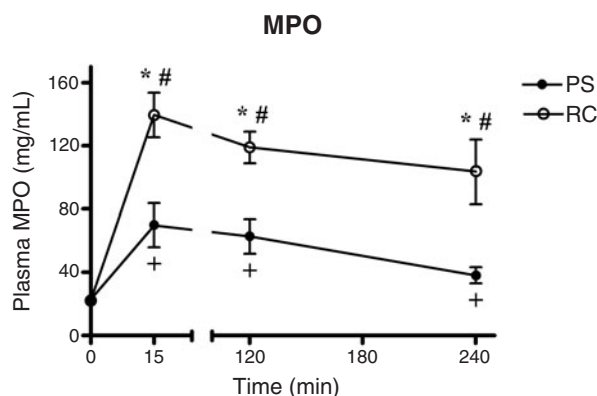


Fig. 2. Changes in MPO in patients using RC and PS membranes at various times during HD. **P* < 0.05 vs PS; ⁺*P* < 0.05 vs PS baseline values; [#]*P* < 0.05 vs RC baseline values.

Effect of dialysis on white blood cells

The changes in white blood cell (WBC) numbers during HD are presented in Table 2. There was a striking decrease in leukocyte and granulocyte counts with RC, which occurred 15 min after the start of treatment. The decrease of leukocyte and granulocyte with PS was much less. The counts at the end of the dialysis session were significantly higher than baseline in the RC membrane group, but not in the PS group.

Effect of dialysis on oxidant production and MPO activity

The levels of O₂⁻ were significantly increased during the first 15 min of dialysis and then decreased with time.

In comparison with the PS group, O₂⁻ levels were higher and significantly different in the RC group at 15 min. The post-dialysis levels at 240 min were lower than baseline for both membranes (Figure 1). However, the intradialysis levels of MPO were significantly increased among the RC and PS groups. The highest level was noted at 15 min and was approximately seven times greater than at baseline. There were significant differences between the two dialysers during the whole session of dialysis (Figure 2). The production of ROS was increased less with synthetic PS membranes.

Effect of dialysis on antioxidant capacity

Erythrocyte SOD and GPx activities were not significantly changed during the HD sessions. There were

also no differences in SOD and GPx levels between the two dialysis membranes at 0, 15, 120 and 240 min. There were no deleterious effects of a dialysis session on antioxidants.

Effect of dialysis on oxidation products

The intradialysis values of the oxidation products were similar to the pre-dialysis values and during the HD sessions, the TBARS levels were not significantly different between the two groups. The levels of AOPP at 240 min were higher than those at baseline in both groups. However, there was no significant difference between the PS and RC membranes. Serum 8-OHdG increased progressively during HD in patients using the RC membrane, but there were no significant differences between the two membranes.

Discussion

The major finding of this study is that HD with RC membranes resulted in significantly increased production of oxidants during a single HD session, whereas dialysis with a PS dialyser had a milder effect. The differences in MPO were significant between the two membranes and persisted throughout the dialysis process. The other markers of oxidant stress did not change significantly and the effects were very slight.

Our results are consistent with the findings that the degree of HD-associated increase in the production of ROS seemed to vary with the use of different membranes [9]. In our study, a novel finding was a significant increase in the production of MPO after a single session of HD using RC membranes in patients on maintenance dialysis. MPO is stored primarily in granulocytes and it accounts for $\leq 5\%$ of total cell protein content in neutrophils. MPO not only holds a central role in microbial killing, but also has been considered an important pathophysiological factor in oxidative stress [15]. Since neutrophils are considered essential effector cells in HD, it is reasonable to assume a contribution of MPO during the process. The difference of the MPO levels between groups exceeds that of the other markers by greater than an order of magnitude and, thus, may serve as a reliable indicator of oxidative stress during single HD sessions using different biocompatible membranes. We also found that dialysis, even with the use of biocompatible PS membranes, contributes to an increase of neutrophil MPO activity. This result suggests that when interpreting the relatively acute changes in oxidative stress during HD, apart from the dialyser, a number of other factors should be considered to contribute to oxidative stress in HD patients, such as the dialysis *per se* and uraemia itself [8].

The effect of various dialysis membranes on activation of complement and alteration of cellular function has been investigated extensively [16]. In this study, we focus on the acute changes by two dialysers with

different biocompatibility. The cellulosic (bioincompatible) membranes induced more significant change of leukocyte counts and ROS production than PS (biocompatible) membranes. In parallel, the ROS production occurred with a similar time course to the leukopenia, which were both considered to be associated with complement activation and the biocompatibility of the dialysis membranes [16]. The excess generation of ROS in HD patients may partially result from activation of peripheral blood cells interacting with the dialyser. Bioincompatibility of the membranes used in HD seems to play a central role in the increased production of oxidative stress.

We found no deleterious effect of a dialysis session on antioxidants. SOD represents the first line of defence against oxidative attack and GPx belongs to the second line of defence. Levels of both SOD and GPx enzymes appear to be unchanged during HD. Although no compensatory changes of antioxidants were noted, the increase of the oxidants and oxidative products may also reflect compromised antioxidant defence. The ability to withstand oxidative stress appeared decreased in HD patients. Thus, it was postulated that their antioxidant capacity was reduced, yet the mechanism of this remains unclear. Therefore, uraemic patients, especially those on maintenance HD, may emerge as a major example of disease-associated oxidative stress because of the excessive production of oxidants accompanied by inadequate antioxidant defences.

The oxidative modification of lipids and proteins have been termed 'long-lived oxidants' to distinguish them from oxygen-derived free radicals whose life span is extremely short [17]. The different life spans may explain why the differences in superoxide anion and MPO production were prominent, whereas those of TBARS, AOPP and 8-OHdG were very slight during a single HD session. Although we have evaluated several biomarkers in this study, the measurement and quantification of oxidative stress remains a difficult issue. We used TBARS, the most commonly used marker, as an index of lipid peroxidation and there were no changes during HD. Similarly, AOPP and 8-OHdG also displayed no statistically significant changes between the two groups. The 'long-lived oxidants', including TBARS, AOPP and 8-OHdG, may not be suitable as oxidative markers during short-term HD sessions.

In conclusion, differences in membrane material and, thus, in the biocompatibility of the dialyser, may play an important role in the imbalance between ROS generation and antioxidant defence during single dialysis sessions. The measurement of MPO may serve as a reliable marker to estimate the degree of oxidative stress during HD using dialysis membranes with different biocompatibilities. However, a limitation of our study was the relatively small number of subjects. Larger randomized studies are needed to confirm our findings.

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Conflict of interest statement. None declared.

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