Effect of haemodiafiltration with online regeneration of ultrafiltrate on oxidative stress in dialysis patients

Lorenzo A Calò, Agostino Naso, Gianni Carraro, Mary Lou Wratten, Elisa Pagnin, Lara Bertipaglia, Mirka Rebeschini, Paul A Davis, Antonio Piccoli and Carmelo Cascone

Abstract

Background. Increased oxidative stress (OxSt) as well as inflammation are risk factors for cardiovascular events and determinant of cardiovascular disease which remains the most common cause of excess morbidity and mortality for end-stage renal disease (ESRD) patients. Haemodiafiltration with on-line regeneration of ultrafiltrate (HFR) has been shown to have a positive impact on markers of inflammation while its effect on OxSt is not known.

Methods. This study evaluates in haemodialysis (HD) patients the effect of HFR on the plasma level of oxidized LDL (OxLDL), a marker of OxSt, and mononuclear cell gene and protein expression of OxSt-related proteins such as p22phox (subunit of NAD(P)H oxidase), PAI-1 (induced by OxSt and atherothrombogenic) and haeme-oxygenase-1 (HO-1) (induced by OxSt). Fourteen patients were randomized into two groups in a crossover design, treated for 6 month periods with HFR (SG8 Plus-Bellco, Mirandola, Italy) or low-flux bicarbonate dialysis (HD) using a polysulphone dialyser 1.8 m². Blood samples were collected at the beginning of the study, after 6 months (crossover) and after 12 months.

Results. ANOVA analysis of the data performed to rule out any crossover effect in either sequence was not significant and thus data from both sequences were combined and then analysed further statistically. HFR reduced mRNA production and protein expression of p22phox and PAI-1 compared with HD (−9 ± 5 vs 2 ± 6 Δ%, P < 0.0001 and −15 ± 20 vs 3 ± 17 Δ%, P < 0.05 for p22phox, −19 ± 6 vs −5 ± 5 Δ%, P < 0.0001 and −24 ± 12 vs 9 ± 15 Δ%, P < 0.0001 for PAI-1). HO-1 was unchanged (−12 ± 8 vs −10 ± 8 Δ% and −21 ± 12 vs −14 ± 8 Δ%) while plasma OxLDL was reduced (−14 ± 19 vs 1 ± 14 Δ%, P < 0.01).

Conclusions. The results of our study indicate that HFR treatment, compared with standard dialysis, has a lower impact on OxSt. Given, the strong relationship between OxSt and inflammation and their impact on the long-term cardiovascular complications in end-stage renal disease patients, HFR might have a more beneficial impact in reducing the risk of atherosclerotic cardiovascular disease in dialysis patients.

Keywords: dialysis; haemodiafiltration; oxidative stress; PAI-1

Introduction

Cardiovascular disease remains the most common cause for the excess of morbidity and mortality in end-stage renal disease patients [1]. Increased oxidative stress (OxSt), inflammation and endothelial dysfunction are recognized non-traditional cardiovascular risk factors in these patients. These patients, in fact, have increased levels of inflammation-related proteins, such as interleukin-6 (IL-6) and C-reactive protein (CRP), as well as OxSt-related proteins, such as NAD(P)H oxidase, which lead to reduced nitric oxide (NO) availability and endothelial dysfunction [2]. The cause(s) of inflammation in dialysis have been shown to be multi-factorial and to include both factors arising from dialysis as well as other non-dialysis-related factors [3,4]. Unfortunately, during the last 20 years the mortality rate in ESRD patients treated with dialysis has remained high, which has prompted the exploration of multiple strategies such as anti-inflammatory treatment using either pharmacological or dialysis-based approaches, to improve outcomes in these patients [5]. A novel dialysis technique, haemodiafiltration with online regeneration of ultrafiltrate (HFR), has recently
been reported to reduce tumour necrosis factor (TNF)-α, IL-6 and CRP [6,7], counteracting the inflammatory state while no data are available on the effect of HFR on OxSt. HFR (double chamber HDF with reinfusion of ultrafiltrate regenerated through a charcoal–resin cartridge), combines the processes of diffusion, convection and adsorbance [8]. During HFR, the ultrafiltrate derived from the convective section of the filter is processed in a charcoal–resin cartridge and the regenerated ultrafiltrate is reinfused into the bloodstream before the diffusive section of the filter. An additional feature is that the resin component of the cartridge adsorbs in particular pro-inflammatory cytokines [6,7].

Given the important role played by OxSt and its associated molecules, including those related to inflammation in increasing cardiovascular disease (CVD) risk in end-stage renal disease (ESRD) [2], HFR may prove useful in reducing the levels of these molecules, and thereby, reduce the morbidity and mortality of dialysis patients. The aim of the current study was to evaluate the effect of dialysis using HFR on OxSt as assessed by plasma markers of OxSt such as oxidized low density lipoproteins (LDL) as well as gene and protein expression levels of OxSt-related proteins such as p22phox, haeme-oxygenase-1 (HO-1) and plasminogen activator inhibitor (PAI)-1 in mononuclear cells.

Patients and methods

Patients

Fourteen patients from the Division of Nephrology II at the Padova University Hospital, eight males and six females, age range 19–60 years, undergoing chronic dialysis treatment (210–240 min three times a week bicarbonate-dialysis) for at least 1 year (range 1–6 years), were recruited and randomized into a 1 year cross-over study. One group of patients was initially treated for 6 months with HFR (SG8 Plus-Bellco, Mirandola Italy) then followed by 6 months of low-flux bicarbonate dialysis with ultrapure dialysate, using a polysulphone dialyser 1.8 m² and another group was first treated with low flux bicarbonate dialysis with ultrapure dialysate, using a polysulphone dialyser 1.8 m² for 6 months followed by 6 months of treatment using HFR. The aetiology of ESRD for the patients was as follows: chronic glomerulonephrites (eight patients); Alport disease (one patient); nephroangiosclerosis (two patients); undiagnosed (three patients). Patients were selected on the basis of the following criteria: the relatively young age, non-smokers, the lack of co-morbidity such as diabetes, chronic obstructive pulmonary diseases, heart failure, cancer, arterio-venous fistula as vascular access and lack of hospitalization in the last 6 months. The reason to select patients with these characteristics comes from the need to show more clear evidence of changes in OxSt and OxSt-related proteins, attributable to the different procedures considered in the study (HFR and standard bicarbonate dialysis), given that the dialysis procedure per se induces OxSt. Blood samples for molecular biology and biochemical determinations have been collected at the beginning of the study, after 6 months (at crossover) and after 12 months.

To minimize the possibility, although not proved, that a different quantitative gene expression between mononuclear cell subtypes could influence the gene expression of the OxSt-related proteins we considered in our study, i.e. through a fluctuation of the number of the different mononuclear cell subtypes, we checked the patients for the absence of changes of biochemical markers of inflammation such as CRP (2.38 ± 1.29 mg/l and 2.57 ± 1.09 mg/l in HFR and HD patients, respectively), α2 globulins (0.68 ± 0.12 g/dl and 0.71 ± 0.09 g/dl in HFR and HD patients, respectively), monocytes (442.9 ± 94.5 × 10^6/1 and 450.9 ± 86.6 × 10^6/1 in HFR and HD patients, respectively), and lymphocytes (1584.3 ± 250.6 × 10^6/1 and 1569.4 ± 272.5 × 10^6/1 in HFR and HD patients, respectively), which counts as well as for no clinical evidence of infectious or inflammatory disease both before the beginning of the study, at cross over and at the end of the study.

Patients’ blood pressure ranged from 135/85 to 150/90 mmHg and anti-hypertensive treatment included calcium channel blockers, ACE inhibitors and β-blockers. All patients were under epoetin treatment at the average dose of 8000 U/week at the beginning of the study ranging from 4000 to 16000 U. In particular, two patients affected by heterozygous thalassemia needed up to 16000 U. During the study, the EPO dose was adjusted in consideration of the haemoglobin level. We have not had significant variations in the EPO dose between low-flux HD and HFR. Haemoglobin level ranged between 11 and 13 mg/dl. Vitamin D, PO₄ binders and calcium supplements were also present in the therapeutic regimen for some patients. None of the patients was under lipid lowering treatment; all patients were treated with supplements of folic acid (10 mg) after dialysis session with no variation throughout the duration of the study.

Single pool KT/V ratio expressed as mean ± SD of KT/V determinations performed every month for all patients was 1.45 ± 0.09 and 1.46 ± 0.13 for HFR and HD patients, respectively.

The study protocol was approved by our institutional authorities and informed consent was obtained from all study participants.

Molecular biology assays

Preparation of mononuclear cells

Peripheral blood mononuclear (PBM) cells were isolated by Ficoll–Paque–PPus gradient (Amersham Pharmacia Biotech, Sweden) from 35 ml of EDTA anti-coagulated blood.

RNA extraction

RNA from PBM cells was extracted using a commercially available kit (RNA Ble, RNA Extraction, Eurobio, Les Vlis, France) with 1 ml of product per ~5 × 10⁶ cells. The extracted RNA had an OD 280/260 ratio between 1.8 and 2.0.
Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR of RNA was performed using the Gene Amp RNA PCR Kit, essentially as described by the manufacturer (Gene Amp RNA PCR Kit, Perkin-Elmer, Foster City, California, USA).

PCR

p22phox gene expression

PCR for p22phox was performed by using specific primers: 5′-3′: TGGCGCGTGGCTTGTGAT GGT (nucleotide sequence position 169–188) and GTTTGTTGTCCCTGCTGGAGT (465–485) designed with the aid of the Primer3 software and using amplification conditions as previously reported [9–11].

HO-1 gene expression

PCR for HO-1 was performed by using specific primers (5′-3′: CAGGCAGAGAATGCTAGTTC (nucleotide sequence position: 79–99) and GCTTCACATA GCGCTGCA (332–349) designed with the use of Primer3 software and using amplification conditions as previously reported [9–11].

PAI-1 gene expression

PCR for PAI-1 was performed by using specific primers (CTCTCTCT GCCCTCACCAAC (932–951) and GTGGAGAGGCT CTTGGTCTG (1143–1124)) designed with the aid of the Primer3 software, and using amplification conditions as previously reported [9–11].

Western blots

HO-1, PAI-1 and p22phox protein expression were assessed using western blot analysis, as previously reported [12,14]. In brief, total protein extracts were obtained by cell lysis with an ice-cold buffer (Tris–HCl 20 mM, NaCl 150 mM, EDTA 5.0 mM, Niaproof 1.5%, Na3VO4 1.0 mM, SDS 0.1%), added with proteases inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). Protein concentration was evaluated by bichinconinic acid assay (BCA Protein Assay, Pierce, Rockford, USA). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Hybod ECL, Amersham, Uppsala, Sweden) and blocked overnight with no-fat milk (5% in Twin-PBS). Membranes were probed with primary polyclonal antibody (Santa Cruz Biotechnologies, SantaCruz, CA, USA) and then HRP-conjugated secondary antibodies (Amersham Biosciences, Uppsala, Sweden) added, and immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, USA).

Protein expression on western blots was quantified using a PC based densitometric semiquantitative analysis using NIH image software, as previously reported [12,14], and were normalized to GADPH, a housekeeping gene.

Oxidized LDL

Oxidized LDL (OxLDL) levels were determined using a commercially available ELISA-based kit (Immundiagnostik AG, Bensheim, Germany). Intra-assay and inter-assay variations of the assay were 5 and 8%, respectively.

Statistical analysis

Data are expressed as percent variations of treatment with HFR and HD versus baseline. Data were evaluated on a Macintosh G4 computer (Apple Computer, Cupertino, CA) using the Statview II statistical package (BrainPower Inc, Calabasas CA, USA). Values were analysed by ANOVA with testing for treatment and sequence effects. P < 0.05 was considered statistically significant.

Results

ANOVA analysis of the data, performed to rule out any crossover effect in either sequence, was not significant and thus data from both sequences were combined and then analysed further statistically. Data are expressed as percent variations (Δ%) of treatment with HFR and HD vs baseline.

Treatment with HFR significantly reduced mononuclear cell p22phox mRNA level and protein expression compared with the treatment with BD: −9 ± 5 vs 2 ± 6 Δ%, P < 0.0001 (mRNA level) and −15 ± 20 vs 3 ± 17 Δ%, P < 0.05 (protein expression), respectively (Figure 1). Mononuclear cell PAI-1 mRNA level and protein expression was significantly reduced by the treatment with HFR compared with the treatment with BD: −19 ± 6 vs −5 ± 5 Δ%, P < 0.0001 (mRNA level)
and $-24 \pm 12 \%$ vs $9 \pm 15 \%$, $P < 0.0001$ (protein expression), respectively (Figure 2).

Treatment with HFR did not modify gene and protein expression of HO-1 compared with the treatment with BD: $-12 \pm 8 \%$ vs $-10 \pm 8 \%$, $P = NS$ (mRNA level) and $-21 \pm 12 \%$ vs $-14 \pm 8 \%$, $P: NS$ (protein expression), respectively. (Figure 3).

Treatment with HFR compared with the treatment with BD significantly reduced the plasma level of OxLDL: $-14 \pm 19 \%$ vs $1 \pm 14 \%$, $P < 0.01$, (Figure 4).

**Discussion**

Patients with renal failure and those under renal replacement treatment with haemodialysis are exposed to OxSt [2,15,16], particularly reactive oxygen species (ROS), which arises from the activation of endothelial cells, reduction of antioxidant systems and increase in prooxidant activity [2]. This increase in ROS not only affects vascular tone via its destruction of vasodilatory NO, but also via its interaction with LDL which forms OxLDL. Both of these events increase the risk of cardiovascular diseases [2]. OxSt in these patients also leads to red blood cell (RBC) membrane lipid peroxidation and RBC destruction, thereby worsening renal failure-induced anaemia. Thus the increased OxSt in patients with ESRD and undergoing dialysis is of considerable concern [2].

A novel dialysis technique, haemodiafiltration with on-line regeneration of ultrafiltrate HFR which combines the processes of diffusion, convection and
adsorbance [8] has been reported to reduce the levels of cytokines such as TNF-α, IL6 and CRP [6,7,17], which are involved in OxSt and inflammatory responses as well as to increase the levels of the anti-inflammatory cytokine IL-10 [17]. This latter study has also suggested that convection can play an important role in the reduction of predialytic levels of inflammatory markers [17]. This study compared HFR and haemodiafiltration (HDF), a technique which combines convection and diffusion, with standard bicarbonate HD. HFR and HDF were both shown to lower the level of pro-inflammatory cytokines compared with standard HD. This, if on one hand underlines the importance of convection for cytokine reduction, on the other establishes the rationale for hypothesizing that HFR could offer a more protective role toward OxSt as well. In fact, while HDF has been associated with increased loss of the antioxidant vitamin C, which is not replaced during reinfusion [18], HFR could be associated with a sparing effect of several water soluble antioxidants that do not have the affinity for the sorbent cartridge. However, a direct effect of HFR on OxSt status has never been tested. The current study was designed to evaluate whether dialysis using HFR compared to the standard 210–240 min, three times a week bicarbonate dialysis had effects on OxSt in dialysis patients. OxSt was assessed by determination of the levels of plasma markers of OxSt as well as by quantitation in mononuclear cells from patients undergoing either HFR or standard bicarbonate HD of gene and protein expression levels of OxSt-related proteins such as p22phox, HO-1 and PAI-1.

The p22phox is a 22 kDa subunit of cytochrome b558 included in the NADH/NADPH oxidase which is present both in leucocytes and in the vascular wall which functions as an integral subunit of the final electron transport from NAD(P)H to haeme and molecular oxygen in generating O2 [19].

In HD patients treated with HFR, the reduction of p22phox mRNA and protein levels not only suggests reduced OxSt, but also, given its presence in leucocytes, an inhibition of leucocyte activation, one of the most well-known causes of OxSt in ESRD. As a consequence, this reduction of p22phox mRNA and protein levels, therefore, suggests an inhibition of OxSt-mediated signaling mechanisms known as responsible for vascular remodeling and atherogenesis induced by ESRD [2,20,21].

Although originally known for the regulation of fibrinolysis, it is now widely recognized that increased PAI-1 is an established OxSt-related response [22].
In fact, overproduction of ROS has been reported to induce PAI-1 gene expression, which was shown to be blocked by the application of antioxidants and scavenging enzymes [22]. Studies have, in fact, characterized the ROS-mediated signaling pathway leading to PAI-1 induction which involves MAPK/PKB, RhoA/Rho kinase and at least three transcription factors such as NF-kB, AP-1 and SP1 [22]. Moreover, PAI-1 production has been linked to inflammatory cytokines such as IL-1, TNF-α, which promote vascular inflammation and atherosclerosis [23]. In addition, the demonstration that OxLDL, the first step of the atherogenic process, induces PAI-1 expression [24] joined with the proatherothrombogenic effect of PAI-1 [23,25], underlines the role of OxSt-mediated PAI-1 production as the key process for induction of atherosclerotic cardiovascular disease, which is the main cause of death in ESRD patients, including, those under renal replacement treatment with HD. Therefore, in our patients treated with HFR, the reduction of PAI-1 mRNA and protein levels also suggests reduced OxSt.

OxLDL is another indicator of OxSt and a cardiovascular risk factor [26]. The results, showing that HFR-treated patients had lower levels of OxLDL, further strengthen the argument that HFR reduces OxSt in these HD patients.

In contrast to the differences between HFR and standard bicarbonate dialysis in terms of other effects on p22phox and PAI-1, no differences were observed in OxSt in these HD patients. Therefore, the reduction of PAI-1 mRNA and protein levels also suggests reduced OxSt.

OxSt is another indicator of OxSt and a cardiovascular risk factor [26]. The results, showing that HFR-treated patients had lower levels of OxLDL, further strengthen the argument that HFR reduces OxSt in these HD patients.

In conclusion, the results of this study indicate that the treatment with HFR has a much lower impact on OxSt, as the levels of expression of proteins related to and the level of markers of OxSt which were lower than those seen with standard dialysis. The more plausible explanation may come from both the efficacy of HFR in reducing the level of pro-oxidant/pro-inflammatory cytokines such as TNF-α, IL-6 [6,7], which are inducers of and involved in the OxSt and inflammatory response, and from a possible sparing effect of HFR on several water soluble antioxidants. Relevant to this possible antioxidant-sparing effect of HFR is the association of HDF, another technique that combines convection and diffusion and shown also to lower pro-inflammatory cytokines [17], with the loss of the water soluble antioxidant Vitamin C, which is not replaced during reinfusion [18]. These effects of HFR might contribute to reduce the oxidative status in dialysis patients in general and explain its effects compared with standard bicarbonate dialysis in reducing the gene and protein expression of the OxSt-related proteins such as those considered in our study. This is further strengthened by the demonstration of the reduced level of OxLDL upon HFR compared with the standard bicarbonate dialysis we have shown in our study and by a possible antioxidant sparing effect of HFR, which, however, still remains to be fully demonstrated.

This lower impact on OxSt suggests that HFR is a more biocompatible system for dialysis. Given the very close relationships between OxSt and inflammation and the determinant role played by OxSt in the induction of inflammation-related mechanisms in ESRD patients, HFR treatment could have considerable clinical impact in reducing the risk of progressive atherosclerotic cardiovascular disease in dialysis patients, which is the main cause of death in these patients [2].

A relevant limitation in our study should be mentioned, which is represented by the small number of selected patients and the fact that the study has been performed in only one single centre. However, if on one hand the pilot nature of the study and the fact that, to our knowledge, this is the first study which uses a molecular biology approach to investigate the effect of HFR on OxSt, might justify the limited number of the patients enrolled, on the other hand the results of this study could serve as a useful working hypothesis for further studies with a larger number of patients enrolled from different dialysis units as well as for extended study durations to allow the benefit of HFR on OxSt/inflammation related complications of dialysis to be conclusively demonstrated.

Conflict of interest statement. M.L.W. is an employee of Bellco Italy.

References


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