Endothelial progenitor cells in patients on extracorporeal maintenance dialysis therapy

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Abstract

Background. Chronic renal failure patients have a high cardiovascular disease burden, low numbers and impaired function of endothelial progenitor cells (EPCs). We hypothesized that enhanced uraemic toxin removal restores EPCs in haemodialysis patients.

Methods. In a prospective, randomized, cross-over trial, 18 patients were subjected to 4 weeks of each low-flux haemodialysis, high-flux haemodialysis and haemodiafiltration differing in uraemic toxin removal. EPCs were determined at baseline and at the end of each 4-week period. A cohort of 16 healthy volunteers served as control. EPCs were studied after culture on fibronectin (CFU-Hill) and collagen-1 (ECFC).

Results. EPC counts and CFU-B cells were lower in maintenance dialysis patients than in healthy controls (P < 0.001) and a reduced fraction of vital ECFCs (P < 0.05), whereas the formation of endothelial cell colonies (ECCs) was increased (P < 0.05). Different middle molecular uraemic toxin removal had no effects on EPC numbers. The number of prototypical EPCs (CD34⁺/VEGFR2-KDR⁺/CD45⁻ ECFCs) was similar between patients and controls. Correlations of plasma C-reactive protein with ECC count, CFU-Hill colony count and CD34⁺/VEGFR2-KDR⁺/CD45⁻ subpopulation of both ECFC and CFU-Hill cells were observed.

Conclusions. Different middle molecule removal has no effect on EPCs. Reduced vitality and enhanced ECC for-
mation suggest growth induction of impaired EPCs in chronic renal failure and are associated with inflammation.

**Keywords:** atherosclerosis; endothelial progenitor cells; end-stage renal disease; haemodialysis; uraemic toxins

**Introduction**

The number of endothelial progenitor cells (EPCs) in blood has been identified as a surrogate biologic marker for vascular function and cumulative cardiovascular risk in the general population [1]. In patients with coronary artery disease, low numbers of EPCs predict the occurrence of cardiovascular events and death from cardiovascular causes [2]. Cardiovascular disease is common in patients on chronic dialysis and represents the principal cause of death in this population. Therefore, the finding that a reduced number of circulating CD34+ cells, a cell population death in this population. Therefore, the finding that a re-

on chronic dialysis and represents the principal cause of cardiovascular events and death from cardiovascular disease, low numbers of EPCs predict the occurrence of cardiovascular events and death from cardiovascular disease [2]. In turn, the initiation of dialysis therapy in uraemic patients with cardiovascular risks and all-cause mortality in patients on maintenance haemodialysis is not surprising [4].

Uraemic toxins inhibit the in vitro differentiation and functional activity of EPCs [3]. Consequently, compared to the general population, patients with chronic renal failure have reduced numbers and an impaired function of circulating EPCs [3,5–7]. These abnormalities aggravate with advancing chronic kidney disease [7]. In turn, the initiation of dialysis therapy in uraemia ameliorates the number of EPCs [3]. The quality of the dialysis treatment may significantly influence EPC quantity and function. In this respect, EPC functions are positively associated with the dialysis dose ($K_{t}/V$) and patients on more frequent, i.e. five to six times per week nocturnal haemodialysis even have re-

stored numbers and function of circulating EPCs compared to conventional haemodialysis patients [5,6]. Furthermore, the levels of uraemic toxins, such as beta2-microglobulin (b2m), inversely correlate with the number of EPCs [8]. Consequently, the switch of patients from high-flux haemo-
dialysis to online haemodiafiltration is considered to result in an amelioration of EPC numbers suggesting a beneficial effect of enhanced middle molecule removal and reduced inflammation triggering effect by more convective and more biocompatible therapy forms [9].

However, most of the present data on EPCs in patients on renal replacement therapy are generated in small, cross-sectional studies with a considerable risk of being biased. Furthermore, there is no uniform definition of EPCs, and several studies must be questioned with regard to the applied methods for the identification of EPCs making the interpretation of their results difficult. To date, there has been no specific unique cell surface marker identified that permits the prospective isolation of an EPC [10].

The purpose of the present study was to verify in a prospective, randomized setting the effects of extracorporeal dialysis procedures differing in middle molecule removal on isolated EPCs as identified by currently acknowledged methods.

**Materials and methods**

**Study design**

The study design was prospective, randomized and cross-over. Study approval was given by the Freiburg Ethics Committee International (registration no. 08/1875).

**Patient characteristics**

Eighteen stable chronic kidney disease stage 5 patients (mean age 60 ± 14 years; 3 female, 15 male) on regular thrice weekly maintenance high-flux haemodialysis were enrolled into the study after they had given written informed consent. Inclusion criteria were age ≥ 18 years, maintenance extracorporeal renal replacement therapy ≥ 3 months, haemocrit > 30%, stable antiaggregation and erythropoiesin regimen, no vascular access-related problems and the absence of malignant and ongoing inflammatory or infectious disease at the time of study enrolment. Exclusion criteria were pregnancy, unstable clinical condition (e.g. cardiac or vascular instability), life expectancy ≤ 512 months, known coagulation problems and participation in another study. Underlying renal diseases were hypertensive nephropathy (n = 5), diabetic nephropathy (n = 3), autosomal dominant polycystic kidney disease (n = 3), glomerulonephritis (n = 2), tubulointerstitial nephritis (n = 2), nephrectomy (n = 2) and post-renal failure (n = 1). The mean duration on dialysis treatment was 46 ± 29 months (range 11–114 months). The body mass index averaged at 27.8 ± 4.3 kg/m². Seventeen patients were hypertensive, 12 had cardiovascular or peripheral vascular disease, six were diabetics and five patients had hypercholesterolemia. The mean daily urine output was 539 ± 719 mL including six patients without residual renal func-
tion. Seventeen patients had efficient native arteriovenous fistulas, and one patient had a patent bi-flux dialysis catheter for blood access. The month before and during the trial period, the patient’s concomitant medications were continued in an unchanged manner including antiaggregation under study conditions. All except one patient received erythropoietin subcutaneously in a weekly average dose of 4500 ± 3400 units (median 4000 units, range 0–15 000 units). Cardiovascular medications included diuretics, beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor antagonists, digitals, calcium channel blockers and vasodilators.

**Study procedures**

Starting with a mid-week session, the patients were randomly assigned to successively receive thrice weekly each 4 weeks of low-flux haemodialysis (referred as LF-HD; low-flux polysulfone, 1.8 m², Fresenius Medical Care AG, Bad Homburg, Germany), 4 weeks of high-flux haemodialysis (referred as HF-HD; high-flux polysulfone, 1.8 m², Fresenius Medical Care AG, Bad Homburg, Germany) and 4 weeks of online predilution haemodiafiltration (referred as HDF; PUREMA® H high-flux polyether-
sulfone, 1.7 m², Membrana GmbH, Wuppertal, Germany) (Figure 1). HD and HDF were performed using Gambro AK 200 S and AK 200 S Ultra monitors (Gambro AB, Lund, Sweden), which prepare ultrapure dialysate. The dialysate flow ($Q_D$) rate was set at 500 mL/min. The infusion flow rate ($Q_I$) in HDF was targeted to be 50% of the dialysate flow rate ($Q_D$). The ultrafiltration flow rate ($Q_{UF}$) of each session was set according to the individual patient’s interdialytic weight gain. Anticoagulation was per-
formed by unchanged adoption of the previous routine heparinization. Sixteen patients received standard heparin and two patients fractionated heparin. For details of the delivered dialysis treatment, refer to Table 1.

**Blood sampling and laboratory procedures**

Blood samples for the laboratory procedures were drawn from the AV fistula immediately after insertion of the arterial dialysis needle before the first session and after completion of each study period. For the measurement of urea and b2m, additional samples were obtained at the end of each first session from the arterial line of the extracorporeal circuit after reduction of $Q_D$ to 50 mL/min for 120 s and the dialysate flow turned off. The respective dialysis doses for urea were quantified by means of the $eKt/V$ [11]. To illustrate differences between the treatment forms with respect to middle molecule removal, the reduction ratio ($RR$) for b2m was calculated as described elsewhere [12].

**Control group**

Blood samples were also obtained from a control group consisting of 16 healthy volunteers (mean age 54 ± 10 years) of both genders (7 female,
9 male) participating in a blood donation programme after signing informed consent. Inclusion criteria were absence of renal impairment, diabetes mellitus and arterial hypertension as well as no history of cardiovascular or peripheral vascular disease. Exclusion criteria were smoking, malignant and ongoing inflammatory or infectious disease.

**Analytical methods**

Urea and phosphate (PO₄) were determined with a Cobas c 111 analyzer (Roche Instrument Center, Rotkreuz, Switzerland). B2m, albumin and high sensitive C-reactive protein (CRP) were measured by laser immunonephelometry (BN ProSpec Analyzer, Dade Behring GmbH, Marburg, Germany).

**Isolation and culture of EPCs**

Peripheral blood mononuclear cells (MNCs) were isolated from heparinized whole blood samples by Ficoll (Ficoll-Paque™ PLUS, StemCell Technologies, St Katharinen, Germany) density-gradient centrifugation. After washing, isolated cells were subsequently resuspended in growth medium and plated in two different ways.

In the first approach described earlier by Hill et al. [1], the cells were resuspended in EndoCult® Liquid Medium Kit (StemCell Technologies, St Katharinen, Germany) and plated on dishes coated with human fibronectin (Becton Dickinson, Heidelberg, Germany). Forty-eight hours after an initial preplating step in a fibronectin-coated plate, the non-adherent cells were collected and replated onto fibronectin-coated plates. After another 72 h, the numbers of colonies were assessed manually by using a contrasting phase microscope (Axioskop 40 CFL, Zeiss, Göttingen, Germany). Colonies of EPCs were identified as multiple thin, flat cells emanating from a central cluster of rounded cells.

According to the approach proposed by Ingram et al. (endothelial colony-forming cells, ECFC) [13], isolated MNCs were resuspended in endothelial growth medium-2 (EGM-2) medium + 10% FCS (Promocell, Heidelberg, Germany) and seeded onto tissue culture plates pre-coated with type 1 rat tail collagen (BD 354400 BioCoat™-6-well plates, Heidelberg, Germany). After 24 h, non-adherent cells and debris were discarded. Adherent cells were washed and then grown with EGM-2 medium, which was changed daily until cell harvest after 7 days. Endothelial cell colonies (ECCs) appeared as well-circumscribed monolayers of cobblestone-appearing cells and were visually enumerated with the contrasting phase microscope.

**Identification of EPCs by fluorescence-activated cell sorting**

EPCs were immunophenotyped and enumerated with a FACScan (Becton Dickinson Biosciences, Heidelberg, Germany) flow cytometer and the CellQuest™ Pro software package (Becton Dickinson). After the supernatant medium and non-adherent cells had been discarded, EPCs were detached from the respective plates with Accutase (PAA, Colbe, Germany) and a three-colour analysis was performed to characterize the co-expression of CD34, CD45 and VEGFR2-KDR. A sample of EPC suspension was incubated with phycoerythrin-Cy5-conjugated CD34 antibodies (PE-
Cy5-conjugated mouse anti-human IgG1, BD Pharmingen™, Heidelberg, Germany), fluoresceinisothiocyanat-conjugated CD45 antibodies (BD Pharmingen™, Maus anti-human, IgG1, Heidelberg, Germany) and phycoerythrin-conjugated VEGFR2-KDR antibodies [mouse anti-human VEGF R2 (KDR)-PE, R&D Wiesbaden-Nordenstadt, Germany]. Blocking experiments with unlabelled antibodies served as controls for the three assays and at least 5000 events were counted per measurement.

In an additional sample, quantitative determination of cells undergoing apoptosis was performed using a ‘Cell Viability Solution’ (7-Amino-Aktinomycin D, BD Via-Probe™, Heidelberg, Germany).

Data analysis
Descriptive analysis of the results was performed by calculating mean values ± standard deviations (SD) or median and range. Within-subject between-treatment differences were analysed by ANOVA and a Tukey post hoc test for normally distributed samples and by Kruskal–Wallis test if normal distribution did not apply. Within-subject within-treatment changes from baseline were assessed using the two-sided paired t-test. For comparisons of patient samples with the control group, a non-paired t-test and a Mann–Whitney U-test were used, respectively. Correlation coefficients were determined for EPCs and pretreatment plasma concentrations of CRP and b2m according to Pearson. A P-value of <0.05 was considered statistically significant. The statistical analysis was performed by means of the ‘Minitab 15 Statistical Software’ package (Minitab, Inc., State College, PA, USA).

Results
None of the patients withdrew from the study and no patient drop-outs were noted.

Treatment data
As a consequence of the online production of infusion fluid (49.5 ± 16.9 L per session), QD in HDF averaged at 315 ± 63 mL/min and was lower (P < 0.001) compared to both HD modes (500 ± 0 mL/min). Treatment differences were also observed for the dialysis dose eKt/V, the b2m RR and the b2m plasma level. For further details, refer to Table 1. In healthy controls, the median of CRP (1.7 mg/L,
Table 2. Cell count of CFU-Hill and ECFC subpopulations normalized for 10⁷ MNC. Medians and ranges are given

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<td>22 097</td>
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<td>CFU-Hill</td>
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<td>75 645</td>
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<td>117 031</td>
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**aP < 0.05. bP < 0.001 vs LF-HD, HF-HD and HDF on days 0 and 28.**
range 0.2–18.0; P = 0.006) and the mean values of b2m (1.7 ± 0.5 mg/L; P < 0.001) and urea (26.0 ± 9.6 mg/dL; P < 0.001) were significantly lower compared to the patients on dialysis.

EPC colony and cell counts

The median of the CFU-Hill colony count in patients on dialysis varied between 5.0 (range 0.0–157.5; HF-HD, day 28) and 35.0 (2.5–262.5; HF-HD, day 0) per 10³ MNC without differences between groups and versus healthy controls [17.5 (0.0–72.5) per 10³ MNC]. In contrast, ECC counts of the dialysis patients [median values between 0.0 (0.0–6.5; HDF, day 0) and 0.8 (0.0–17.5; HDF, day 28) per 10³ MNC] were significantly (P < 0.05) higher compared to the controls [0.0 (0.0 to 0.3) per 10³ MNC], in which ECFC colony formation was virtually nonexistent (Figure 2).

The medians of the total CFU-Hill cell counts in the treatment groups ranged between 801 × 10³ (265 × 10³ to 1281 × 10³; LF-HD, day 28) and 1080 × 10³ (110 × 10³ to 2079 × 10³; HF-HD, day 0) per 10⁷ MNC. In healthy controls, it was 1620 × 10³ (213 × 10³ to 2659 × 10³) per 10⁷ MNC without being significantly different from the dialysis patients (P = 0.079). The total ECFC counts were also not different between dialysis treatment groups [from 5.4 × 10⁷ (0.2 × 10⁷ to 101 × 10⁷; LF-HD, day 28) per 10⁷ MNC to 12.7 × 10⁷ (0.9 × 10⁷ 60.7 × 10⁷; LF-HD, day 0) per 10⁷ MNC] but were much lower (P < 0.001) compared to the controls [75.9 × 10³ (32.0 × 10³ to 318 × 10³) per 10⁷ MNC] (Figure 3).

EPC subpopulations

Subpopulation counts of CFU-Hill cells and ECFC were similar between dialysis modes (Table 2). Significant differences were only determined versus the healthy controls for ECFC subpopulations with the immunophenotypes CD34+/VEGFR2-KDR+/CD45− and CD34+/VEGFR2-KDR−/CD45−. Particularly for CD34+/VEGFR2-KDR+/CD45− ECFC, which are currently regarded as prototypical for EPCs, differences were found neither between treatments nor versus controls (Table 2).

The proportion of vital CFU-Hill cells was similar between dialysis patients (93 ± 3%, HDF, day 0, to 96 ± 3%, HDF, day 28) and the control group (97 ± 4%). In contrast, the fraction of vital ECFC was higher (P < 0.05) in controls (86 ± 9%) compared with the dialysis patients which presented a large variation in the degree of apoptosis without differences between the treatment modes and a cell vitality between 54 ± 25% (LF-HD, day 28) and 68 ± 20% (HF-HD, day 0).

EPCs, inflammation and uraemic toxins

CRP in plasma highly correlated (r = 0.556; P < 0.001) with the ECFC colony count (Figure 4A) and was also linearly associated with the CD34+/VEGFR2-KDR+/CD45− subpopulation of CFU-Hill cells (r = 0.372; P < 0.001) and ECFC (r = 0.364; P < 0.001) (Figure 4B). Pretreatment urea and b2m inversely correlated with the CD34+/VEGFR2-KDR−/CD45− subpopulation of ECFCs (r = −0.419; P < 0.001 and r = −0.212; P = 0.034, respectively). A negative linear association was also determined for the CD34+/VEGFR2-KDR+/CD45− subpopulation of ECFC (r = −0.381; P < 0.001).

Discussion

In maintenance dialysis patients, cardiovascular disease is common and represents the most important cause of death [14,15]. Although the HEMO study was unable to demonstrate an overall beneficial effect of high-flux dialysis on survival, patients on this therapy form had a lower relative risk for cardiac death compared to low-flux dialysis [16]. Furthermore, a post hoc analysis of the 4D data indicated significant advantages of increased middle molecule removal with respect to survival and to a combined cardiovascular endpoint [17]. Therefore, the concept that an enhancement of uraemic toxin removal by dialysis treatment may restore EPC function and, finally, improve cardiovascular outcome is tempting. In this respect, the results of the present study are disappointing. Despite a clear sep-
EPCs in maintenance dialysis patients

George et al. identified circulating EPCs based on the co-expression of CD34, VEGFR2-KDR and CD133 [8], which are highly enriched of hematopoietic progenitor cells but fail to specifically isolate ECFC. These cells are involved in neoangiogenesis and are predictive biomarkers for some human diseases, such as coronary artery disease [20]. In their cross-sectional analysis, Jourde-Chiche et al. observed that the number of these cells inversely correlated with the level of b2m [8]. Even if we failed to demonstrate a beneficial impact of higher middle molecular toxin removal on EPCs, our data confirm these results. Lower levels of both the surrogate marker middle molecule b2m and the small solute urea were weakly linearly associated with higher numbers of several CFU-Hill and ECFC subpopulations, among them, the CD34+/VEGFR2-KDR+/CD45- ECFC progeny regarded as true EPCs. The lacking effect of enhanced toxin removal by the more convective dialysis procedures, i.e. HF-HD and especially HDF, does not contradict this association because the level of uraemic toxins their vitality is impaired leading to enhanced apoptosis. Since after 24 h non-adherent cells were always discarded, including particularly those being apoptotic, the total number of ECFCs after 7 days in culture was lower. Previous studies have shown that in vitro EPC differentiation and functional activity are inhibited and apoptosis of EPCs is enhanced by uraemic serum [3,8], but our in vitro approach was completely different. However, the assumed state of ineffective ECFC maturation requires confirmation. Furthermore, it must be underlined that for prototypical EPCs, i.e. ECFCs with the immunophenotype CD34+/VEGFR2-KDR+/CD45-, differences were found neither between treatments nor versus controls. Significant differences were only determined for the CD34+/VEGFR2-KDR+/CD45- and CD34+/VEGFR2-KDR+/CD45- ECFC subpopulations. Cells co-expressing CD45 behave similar to macrophages, which are well known to express 'endothelial specific' proteins and to participate in regulatory form in neoangiogenesis [10].

Inflammation has been shown to be strongly associated with atherosclerosis and cardiovascular outcome in chronic renal failure [27,28]. Our data demonstrated a strong correlation of the plasma CRP level with the ECC count ($r = 0.556; P < 0.001$). CRP was also linearly associated with the CD34+/VEGFR2-KDR+/CD45- subpopulation of both ECFCs ($r = 0.364; P < 0.001$) and CFU-Hill cells ($r = 0.372; P < 0.001$). Disregarding the methodological differences with respect to previous trials and few confounding inflammatory incidents occurring over the study period in some patients, such an association has not been observed before in patients with chronic renal failure.

Only in the small study by George et al. on subjects with angina pectoris syndromes, a positive correlation of CRP levels and the number of circulating EPCs was reported suggesting that a systemic inflammatory state could potentially facilitate peripheral endothelial precursor mobilization [26]. Although the role of EPCs in coronary artery disease is not fully elucidated [20,29], further evidence for the crucial role of inflammation in the biological behaviour of blood-derived EPCs comes from in vitro data showing that IL-6 receptors are expressed in EPCs and that administration of IL-6 stimulated EPC proliferation, migration and tube formation in a dose-dependent manner [30].
In this regard, other in vitro experiments have produced conflicting results which indicate that EPCs isolated from blood of healthy volunteers are concentration-dependently inhibited in differentiation, survival and function, and present increased apoptosis by CRP added to culture [31–33]. However, methodological differences give rise to doubts if these results are directly applicable to our findings.

Conclusions

The concept of a favourable effect of enhanced middle molecule removal by convective extracorporeal renal replacement therapy on EPCs is not supported by the results of the present study. A reduced vitality of EPCs and enhanced ECC formation in maintenance dialysis patients suggest growth induction of impaired EPCs in chronic renal failure. In this respect, inflammation, which is associated with atherosclerosis, seems to play an important role in the biological behaviour of blood-derived EPCs besides potentially other local and systemic stimuli.

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Conflict of interest statement. D.H.K. has received lecturer fees from Baxter Healthcare and Membrana GmbH. H.D.L. is a full-time employee of Membrana GmbH, Wuppertal, Germany. C.W. serves on the BAXTER European Renal Advisory Board for peritoneal dialysis and received lecturer fees from Fresenius Medical Care. B.C. has received fees for lectures from Fresenius Medical Care, Amgen, Roche and Janssen-Cilag.

References

32. Suh W, Kim KL, Choi H et al. C-reactive protein impairs angiogenic functions and decreases the secretion of arteriogenic chemo-
Arterial stiffness and functional properties in chronic kidney disease patients on different dialysis modalities: an exploratory study

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Abstract

Background. Abnormalities of vascular function and accumulation of oxidative stress have been associated with chronic kidney disease (CKD). Dialysis modalities, peritoneal dialysis (PD) and haemodialysis (HD) may differentially impact on vascular function and oxidative stress. Methods. Patients undergoing living donor transplantation were studied for vascular stiffness using pulse wave velocity measurements, and inferior epigastric arteries were harvested to examine in vitro stiffness and functional properties and evidence of oxidative stress. Forty-one patients were studied representing PD (n = 12), HD (n = 14) and non-dialysed recipients (n = 15). Results. We demonstrated differences in stiffness from in vivo and in vitro measurements such that non-dialysis < HD < PD groups. The stiffness measurements did not correlate with duration of CKD nor dialysis duration, but did so with phosphate levels (r = 0.356, P = 0.02). From the in vitro isometric force experiments, HD arteries demonstrated decreased contractility and endothelium-dependent relaxation compared with PD and non-dialysis vessels. Level of oxidative stress (as indicated by the 8-isoprostane level) was 30% higher in HD arteries than in PD arteries. Protein expression of inducible nitric oxide synthase, NADPH subunits and xanthine oxidase was upregulated in HD arteries, while superoxide dismutase was downregulated. The compromised vascular function in HD arteries was improved by pharmacological means that eliminated oxidative stress. Conclusions. We report associations between vasomotor function and oxidative stress in the vasculature of patients receiving different dialysis therapies. Oxidative stress, which may be differentially augmented during PD and HD, may play an important role in the vascular dysfunction in dialysis populations.

Keywords: chronic kidney disease; haemodialysis; oxidative stress; peritoneal dialysis; vasomotor function

Introduction

Chronic kidney disease (CKD) is a well-recognized risk factor for cardiovascular disease (CVD). Furthermore, the leading cause of death in both CKD and dialysis populations remains to be CVD [1,2]. There is increasing evidence that the cardiac and vascular abnormalities develop early in CKD and become more severe as the disease progresses to end stage. The complex process of vascular calcification and the consequent arterial stiffening have received substantial attention in all CKD populations. However, whether and how different dialysis modalities might impact on the vascular function has not been the focus of much study.

Vascular dysfunction is characterized by aberrations in endothelial secretion, smooth muscle contractility and mechanical property. Increased arterial stiffness results in elevated left ventricular stress and reduced coronary perfusion, and the subsequent hypertrophy and cardiomyopathy predispose to congestive heart failure and sudden death in dialysed CKD patients [1–3]. Endothelial dysfunction has been described in the vasculature of patients on both peritoneal dialysis (PD) and haemodialysis (HD), and is implicated as the initial pathological step in the progression of vascular damage [4,5]. Elevated production of vasoconstrictors, such as norepinephrine, endothelin-1 and angio-