Endothelial dysfunction in patients with kidney failure and vascular risk factors: acute effects of hemodialysis

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Blood flow; Nitric oxide; Vasoconstriction; Vasodilation.

Background. **Patients with kidney failure present endothelial dysfunction, which was shown to be partly corrected by hemodialysis. No data exist on the effects of hemodialysis on endothelial dysfunction in kidney failure patients with associated vascular risk factors. The aim of this study was to evaluate the acute effects of hemodialysis on endothelial dysfunction in patients with kidney failure and associated vascular risk factors and to assess the role of endothelium-toxic substances.**

Methods. **We assessed endothelial dysfunction in 13 patients with chronic renal failure and other vascular risk factors before and after hemodialysis and in 13 healthy controls and simultaneously measured nitric oxide (NO) synthesis and activity. Endothelial dysfunction was studied using an echographic method as flow-mediated dilation (FMD) of the brachial artery; plasma NO_2^- and NO_3^-, cyclic guanosine-5-monophosphate (cGMP), plasma homocysteine levels and low molecular mass-advanced glycation end-products (LMM-AGEs) were simultaneously measured.**

Results. **As compared with healthy controls, patients with renal failure showed a reduced FMD (2.89 ± 1.43 vs $7.81 \pm 1.54\%$, $p < 0.01$) which was not corrected by dialysis (after dialysis $2.40 \pm 1.65\%$, p = NS vs pre). Plasma NO_2^- and NO_3^- were normal or slightly increased and remained unchanged after dialysis. Plasma cGMP levels were reduced and remained unchanged after dialysis. Homocysteine and LMM-AGE plasma levels were raised and, although significantly reduced by dialysis, remained higher than in controls.**

Conclusions. **Patients with kidney failure and associated vascular risk factors show an endothelial dysfunction related to defective NO activity, which is not corrected by hemodialysis despite the reduction, though not to normal, in homocysteine and LMM-AGE levels. Endothelial dysfunction may contribute to the progression of atherosclerosis in patients with kidney failure and vascular risk factors.**

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Introduction

Kidney failure is associated with accelerated atherosclerosis and a greatly enhanced incidence of ischemic cardiovascular disease[1].

Endothelium-derived mediators, amongst which nitric oxide (NO) has a prominent role, exert antiatherosclerotic effects by preventing platelet adhesion and aggregation, leukocyte adhesion, and smooth muscle cell proliferation[2,3]. NO is a very labile mediator produced by the endothelium after stimulation by soluble agonists and by high shear forces. NO diffuses to the underlying smooth muscle cells and it is released into the vessel lumen where it stimulates the soluble guanylate cyclase of the target cells to generate cyclic guanosine-5-monophosphate (cGMP), the latter being responsible for smooth muscle relaxation, and thus endothelium-dependent vasodilation, and for platelet and leukocyte inhibition.

Endothelial dysfunction, and in particular the loss of endothelium-dependent vasodilation, is an early marker of the atherosclerotic degeneration of medium-sized arteries[2-4] and it has been shown to be associated with a number of risk factors, such as smoking[5,6], hypertension[7], diabetes[8], hypercholesterolemia[9] and hyperhomocysteinemia[10], typically leading to atherothrombotic complications. Interestingly enough, the correction of risk factors by dietary and pharmacologic means or lifestyle changes is able to restore, in part or totally, endothelial dysfunction[4,8,9,11] and some measures, such as the acute administration of antioxidants (vitamin C), may rapidly correct the reduced endothelial function[11].

Endothelial dysfunction is typically present in patients with kidney failure[12-15]. This condition is associated with the accumulation in blood of a series of substances potentially toxic to the endothelium, such as homocysteine[16], low molecular mass-advanced glycation end-products (LMM-AGEs)[17] or others[18]. It is thus conceivable that an efficient hemodialytic procedure, by removing accumulated toxic substances from blood, may reverse the endothelial dysfunction typically present in uremic patients. Should this be so, then a more strict hemodialytic control could be associated with a lower long-term incidence of cardiovascular events.

Previous studies have assessed endothelial dysfunction in uremic patients and the impact of dialysis on it with positive results[19,20], although contrasting data have been reported[21]. However, most of these studies were carried out in uremic patients without other conditions (vascular risk factors or a history of vasculopathy) known to be associated with endothelial dysfunction[20,21]. The majority of uremic patients, however, do have associated cardiovascular risk factors or signs of atherosclerosis and no information exists on the effects of dialysis on endothelial function in these patients. Indeed, the response to endothelium-protective measures may well be different in vasculopathic patients with associated kidney failure as shown by the lack of any effect of folic acid or L-arginine[16,22], which instead restore endothelial function in patients with vasculopathy and normal renal function. On the other hand, endothelium-toxic substances, such as homocysteine or AGEs, accumulate both in kidney failure and in atherosclerosis[23,24], and it is thus conceivable that their removal by dialysis[21,25] may improve endothelial function in both conditions. To the best of our knowledge, no studies have simultaneously evaluated the effects of hemodialysis on endothelium-mediated vasodilation and on the plasma levels of potential endothelium-toxic substances in patients with kidney failure and atherosclerosis, which actually represent the majority of patients with long-lasting uremia. Thus, the aim of our work was to determine whether dialysis, by removing endothelium-toxic substances, improves endothelial function in uremic patients with associated atherosclerosis. To this end, flow-mediated dilation (FMD), a sensitive parameter of NO-mediated endothelial function[4,26], plasma NO_2^- and NO_3^-, an index of endogenous NO production[13,27], plasma cGMP, an index of the biologic activity of endogenous NO[28], and homocysteine and LMM-AGE plasma levels were measured before and after dialysis.

Methods

Study design. Thirteen patients with chronic renal failure (9 males, 4 females, mean age 55.6 ± 13.0 years, range 40-74 years) were enrolled in the study. All patients had to be on chronic, periodic hemodialysis since at least 6 months. The average time on dialysis was 7.1 ± 4.2 years (range 2-17 years); all were receiving hemodialysis 3 times a week with one of the following membrane dialyzers: cuprophan, cellulose acetate, polyacrilonitrile, polymethylacrilate.

The causes of renal failure included glomerulonephritis (n = 7), pyelonephritis (n = 2), diabetic nephropathy (n = 2), hypertensive nephropathy (n = 1), and connective tissue disease (n = 1). One patient had a history of cerebrovascular disease, 3 of peripheral vascular disease, and 1 of ischemic heart disease. All subjects were either current (n = 4) or previous smokers (n = 9), 6 had hypertension, 2 had type 2 diabetes, and 3 hypercholesterolemia. Concomitant treatments were ACE-inhibitors (n = 4), clonidine (n = 1) atenolol (n = 1), nifedipine (n = 1), diazoxide (n = 1), furosemide (n = 1), and glibenclamide (n = 1). All but 2 were on erythropoietin treatment (2000-4000 U/week).

Concomitantly, 13 control subjects (6 males, 7 females, mean age 48 ± 11.8 years, range 24-60 years), with no known history of cardiovascular disease or hyperlipidemia, diabetes, hypertension and smoking, were studied.

All subjects gave informed, written consent to participate in the study. The study was carried out in conformation with the principles outlined in the declaration of Helsinki.

Each patient was studied under fasting conditions in the morning, 30-45 min before the beginning of the hemodialytic procedure, and again 30-45 min after the end of hemodialysis, for endothelial function; blood sampling was carried out for biochemical studies immediately before and at the end of hemodialysis.

Control subjects were studied in the morning under fasting conditions, undergoing endothelial function studies first and blood sampling, on the contralateral arm, at the end of the procedure.

Endothelial function studies. FMD was studied, as previously described[6], by the non-invasive method of Celermajer[4,26]. Briefly, the diameter of the brachial artery, of the arm not carrying the arterio-venous fistula for renal insufficiency patients (mainly the right arm) and of the right arm for healthy controls, was measured by B-mode ultrasonography with a 7.0 MHz linear-array transducer and an Acuson 128 XP/5 System (Acuson, Mountain View, CA, USA) at a location 2 to 5 cm above the elbow. The basal diameter of the artery was measured with the patients lying in the supine position, after 10 min of rest; then, a sphygmomanometer cuff placed on the forearm, was inflated to 240 mmHg for 3 min, the cuff was released and, 45 s after peak reactive hyperemic blood flow, the arterial diameter was measured again. The transducer was kept in the same position throughout the procedure and a cutaneous marker was placed in order to allow examination of the same part of the artery on the post-dialysis study. The center of the artery was identified when the clearest picture of

the anterior and the posterior vessel wall layers was obtained. All the studies were carried out by the same operator.

Depth and gain settings were set to optimize the lumen/arterial wall interface, and the machine-operating parameters were not changed at any time during the study. All the ultrasound scan data were recorded on super VHS video for later analysis.

Analysis of the scan data was carried out by another operator who was blinded to the subjects and phase (before or after dialysis) of the study. Measurements were taken from the anterior to the posterior "m" line at end diastole, incident with the R-wave on a continuously recorded ECG, and calculated on the average of four consecutive cardiac cycles. The arterial blood flow velocity (time averaged maximal flow velocity) was measured using a pulsed Doppler signal at a 70° angle to the vessel with the range gate (1.5 mm) in the center of the artery. The peak increase in blood flow velocity was measured as the maximum velocity in a single cardiac cycle within the first 15 s of cuff release and was expressed as a percentage of the baseline velocity, as a quantitative estimate of reactive hyperemia[14]. In each session, before endothelial function studies, the systolic and diastolic blood pressure (sphygmomanometer) and heart rate (from continuous ECG recording) were measured.

Biochemical measurements. NO synthesis was evaluated by measuring the levels of NO_2^- and NO_3^- (the stable metabolites of NO) in plasma. Plasma samples were thawed in ice and diluted with an equal volume of water. Proteins were removed by ultra filtration (Centricon 10 concentrators, Amicon Inc., Beverly, MA, USA) at 5000× rpm for 30 min.

To determine the NO_2^- concentration in the plasma ultra filtrate, the fluorimetric reaction of nitrite with hydralazine and high-performance liquid chromatography (HPLC) determination of the tetrazolophthalazine derivative were used. In brief, a 100 μl aliquot of ultra filtrate was mixed with an equal volume of hydralazine solution (0.5 mg/ml in 1 MHCl containing 5 mM mercuric chloride) and incubated at 37°C for 30 min. Fifty μl of the mixture were directly injected into the chromatographic apparatus (Jasco Europe, Milan, Italy) and analysis was performed using a Spherisorb ODS2, 5 μ, 0.4 × 15 cm column, equilibrated and isocratically eluted with 0.05 M potassium dihydrogen phosphate solution/20% acetonitrile (pH 4.5) at a flow rate of 1 ml/min. Analyte detection was carried out by a model 821-FP Jasco spectrofluorimetric detector set at 228 nm ex/360 nm em. Quantification was achieved by using external standard calibration and the data were expressed as μmol/l.

In accordance with the recommendations of Curtis et al.[29], the NO_3^- concentration in the plasma ultra filtrate was determined using ion-chromatography with UV detection at 214 nm and expressed as μmol/l.

The concentration of LMM-glycated peptides in the plasma ultra filtrate was determined using the method described by Floridi et al.[30] and expressed as arbitrary units (AU/ml).

Aliquots for plasma homocysteine assessment were placed on ice, centrifuged within 1 hour of collection, and the separated plasma stored at -20°C before assays. Analysis of the total homocysteine in plasma was performed using the HPLC methodology described by Ubbink et al.[31] and the data expressed as μmol/l.

Analytical grade chemicals were purchased from SIGMA (Milan, Italy). HPLC grade water was used.

A commercial kit (EIA) (Amersham Pharmacia Biotech, Little Chalfont, UK) was used to measure plasma cGMP levels. Briefly, plasma from citrated blood was separated immediately after blood collection by centrifugation and then acetylated with a mixture containing triethylamine and acetic anhydride. Acetylated samples were then transferred in the microtiter plate provided by Amersham Pharmacia Biotech, and, as recommended by the manufacturer, the assay carried out following the protocol for total cellular cGMP for the low curve range (2-512 fmol/well); data are expressed as pmol/ml.

Statistical analysis. The study size was calculated on the basis of power estimates derived from previous studies on the variability in FMD measurements[32]. It was expected that in the hemodialysis patients, FMD would be reduced, as compared with the control subjects, to a value approximately one third of normal[12-14] (normal value 9.0 ± 1.5%). It was estimated that the attainable increase with hemodialysis could be an average of approximately +2%, taking as a reference the improvements observed in previous intervention studies[4,11] and considering this as the minimum change required to establish a treatment benefit[32]. Assuming an 80% power (β-error = 0.2) and an α-error of 0.05, it was calculated that a sample size of 13 subjects would be required to detect a significant FMD improvement by dialysis.

Two-way analysis of variance was applied for the comparison of data before and after dialysis and one-way analysis of variance for all other tests, followed by Tukey's test for multiple comparisons. Data are expressed as means ± SEM.

Results

Endothelial function studies. Before dialysis, FMD in kidney failure patients was significantly lower than that of age-matched, healthy controls (2.89 ± 1.43 vs 7.81 ± 1.54%, $p < 0.01$). Even when dropping from the patients' group the 3 cases aged > 65 years (therefore considering only 10 patients with an average age of 49.4 ± 9.5 years; 6 males) the impairment of FMD as compared with controls remained highly significant (FMD

patients 2.95 ± 1.69%, p < 0.05 vs controls), thus excluding an age or sex effect in the difference observed.

The FMD did not significantly change after dialysis (2.40 ± 1.65%, p = NS vs pre) (Fig. 1). Even when analyzed separately depending on the dialysis membrane used, no consistent improvement in FMD was seen following dialysis.

The basal omeral blood flow before dialysis was 413 ± 41 ml/min and the peak maximal reactive hyperemia flow was 363 ± 21% of basal. The corresponding values for healthy controls were 381 ± 76 ml/min and 450 ± 51%, which were not significantly different.

After the dialytic procedure, the basal flow was 431 ± 49 ml/min and the reactive increase 334 ± 25% of basal (p = NS vs pre).

Systolic and diastolic blood pressures in kidney failure patients before dialysis were 158 ± 6.9 and 86 ± 2.8 mmHg respectively, and after dialysis these were reduced to 131 ± 8.4 (p < 0.0013 vs pre) and to 77.5 ± 2.7 mmHg (p < 0.0007 vs pre) respectively. Heart rate was 79.4 ± 2.9 b/min pre-dialysis and 89.6 ± 4.0 b/min after dialysis (p < 0.004 vs pre). No differences between the different dialyzer membranes were observed concerning the effects on endothelial function.

Biochemical measurements. In kidney failure patients, before dialysis plasma nitrite and nitrate levels were slightly higher (significantly for nitrites) than those observed in healthy controls; dialysis reduced, though not significantly, both metabolites in plasma (Table I). Before dialysis, plasma cGMP levels were significantly lower in kidney failure patients than in healthy controls and hemodialysis further reduced them, although not significantly (Table I).

Plasma homocysteine levels were significantly higher in kidney failure patients than in healthy controls, both before and after hemodialysis; the dialytic procedure significantly reduced plasma homocysteine levels (Fig. 2).

Plasma LMM-AGE levels were strikingly, and significantly, higher in kidney failure patients than in healthy controls, both before (p < 0.011) and after (p < 0.008) hemodialysis. The dialytic procedure reduced the plasma levels of LMM-AGEs (p < 0.05 vs pre-dialysis) by half. In spite of this their levels were still more than 20 times higher than control values (Fig. 3).

An assessment of the correlation between the biochemical parameters and FMD in kidney failure patients, before and after hemodialysis, was carried out. No correlations were evident except for a weak positive correlation between FMD and NO_2^- levels ($r^2 = 0.18$, n = 26, p < 0.03). In particular, plasma LMM-AGE levels and FMD in kidney failure patients did not show any correlation. Moreover, no significant correlations were found between plasma homocysteine and LMM-AGE levels or between these parameters and NO_2^- and NO_3^- or cGMP levels.

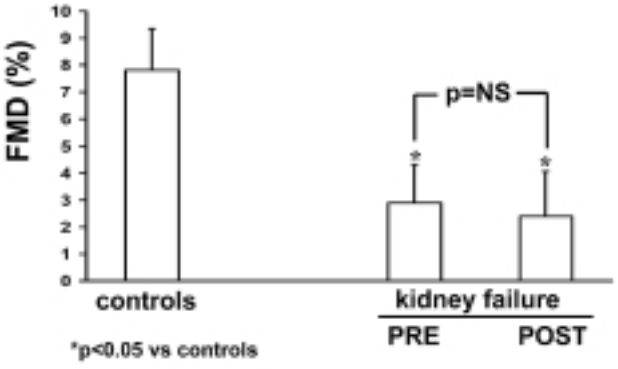

Figure 1. *Flow-mediated dilation (FMD) in healthy controls and in kidney failure patients, before (pre) and after (post) hemodialysis.*

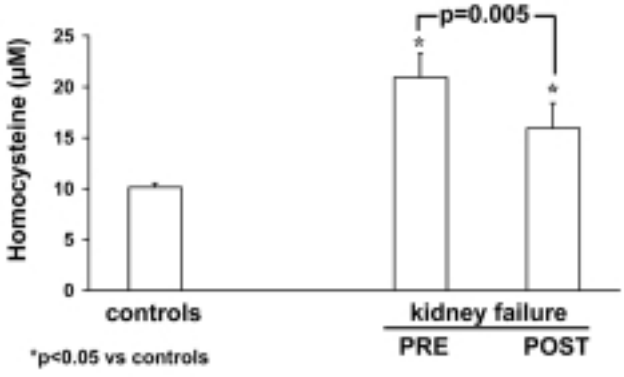

Figure 2. *Plasma homocysteine levels in healthy controls and in kidney failure patients, before (pre) and after (post) hemodialysis.*

Table I. NO_2^-, NO_3^- and cyclic guanosine-5-monophosphate (cGMP) levels in controls and in kidney failure patients, before and after dialysis.

	Controls	Kidney failure		p	
		Pre-dialysis	Post-dialysis	Pre-dialysis vs control	Pre-dialysis vs post-dialysis
NO_2^- (µmol/l)	0.7 ± 0.3	0.9 ± 0.2	0.8 ± 0.1	NS	NS
NO_3^- (µmol/l)	67.8 ± 8.4	104.4 ± 12.1	91.1 ± 9.1	< 0.05	NS
cGMP (pmol/ml)	8.0 ± 0.31	4.21 ± 0.338	3.8 ± 0.29	< 0.01	NS

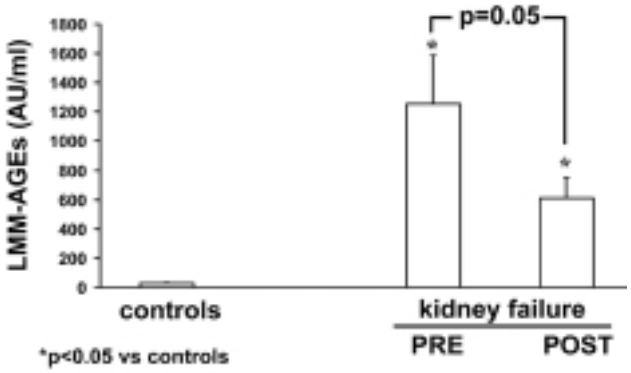

Figure 3. *Low molecular mass-advanced glycation end-products (LMM-AGEs) in healthy controls and in kidney failure patients, before (pre) and after (post) hemodialysis.*

Discussion

Our data confirm that endothelium-dependent vasodilation, a parameter of vascular function largely dependent on endothelial NO production, is severely impaired in kidney failure patients with vasculopathy and/or associated risk factors for atherosclerosis. The fact that some of our patients were under chronic treatment with drugs potentially able to improve endothelial function, such as ACE-inhibitors[33], further emphasizes the observed degree of impairment of NO-mediated endothelial function. NO is considered to be an endogenous protective factor against ischemic cardiovascular disease[2,3] and, indeed, NO biosynthesis is reported to be reduced in patients with kidney failure[13,27,34] as well as in patients with risk factors for atherosclerosis or with ischemic cardiovascular disease[3].

The acute impact of hemodialysis on NO production is rather controversial[35-39]. Recently Cross et al.[22] have shown a striking increase in FMD after dialysis; however, in their series (16 patients), patients with associated causes of endothelial dysfunction (cigarette smoking, diabetes, hypercholesterolemia) were excluded. Our data confirm the presence of an endothelial dysfunction of conduit arteries in patients on chronic hemodialysis and associated atherosclerosis, accompanied by the accumulation in blood of a series of potential endothelium-toxic substances. However, NO_2^- and NO_3^-, an expression of endogenous NO biosynthesis, were increased and despite this, cGMP, an expression of the NO biological activity[28], was reduced. These data suggest that the functional response to NO, rather than NO biosynthesis, is altered in chronic hemodialysis patients with associated vascular risk factors, a conclusion supported by previous observations[40].

In these patients, the dialytic procedure, while significantly reducing plasma levels of homocysteine and LMM-AGEs, did not increase FMD and, correspondingly, slightly reduced NO biosynthesis and plasma cGMP. This effect may be the consequence of the inactivation of endogenous NO by the generation of reactive oxygen species, as shown by Miyazaki et al.[21] in a study on 12 patients undergoing dialysis either with conventional or with vitamin E-coated membranes.

Thus, the removal of dialyzable endothelium-toxic substances is not sufficient to correct the impaired FMD in these patients. Indeed, even slightly elevated levels of homocysteine are able to produce an impairment of endothelial function[10] and, in fact, homocysteine levels after hemodialysis remained above the values observed in controls. In agreement with this is the recent observation that folic acid treatment of patients with predialysis renal failure, although reducing plasma homocysteine levels to values similar to those observed post-dialysis in our study, does not affect the altered FMD[16].

Similarly, hemodialysis drastically reduced LMM-AGE levels, but in agreement with previous results[25], these remained far higher than those observed in controls. The persistence of elevated LMM-AGE levels may contribute to the lack of any significant effect of hemodialysis on endothelial dysfunction in patients with kidney failure and associated atherosclerosis and may contribute to the rapid progression of vascular disease[1,41]. The accumulation of other substances potentially interfering with endothelial function and not completely removed by dialysis, such as asymmetrical dimethylarginine[18], cannot be excluded.

Very few studies have assessed the impact of hemodialysis on NO-mediated vasodilation and none in patients with associated atherosclerotic disease or risk factors. Our data show that hemodialysis, although largely able to remove endothelium-toxic substances, does not ameliorate to any significant extent endothelial function in uremic patients with associated atherosclerosis.

It remains to be established whether more efficient dialytic procedures (daily dialysis and/or highly efficient membranes) or chronic antioxidant treatment[42] may reverse the endothelial dysfunction of kidney failure patients with associated atherosclerosis to an extent similar to that previously demonstrated in patients with kidney failure and no other causes of vascular disease.

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