Uremic serum inhibits *in vitro* expression of chemokine SDF-1: impact of uremic toxicity on endothelial injury

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ABSTRACT

Introduction: Endothelial dysfunction is important in the pathogenesis of cardiovascular disease (CVD) related to chronic kidney disease (CKD). Stromal cell-derived factor-1 (SDF-1) is a chemokine which mobilizes endothelial progenitor cells (EPC) and together with interleukin-8 (IL-8) may be used as markers of tissue injury and repair. **Objective:** This study investigated *in vivo* and *in vitro* the effect of uremic media on SDF-1 and IL-8 expression. **Methods:** Systemic inflammation was assessed by C-reactive protein (CRP) and interleukin-6 (IL-6). IL-8 and SDF-1 were measured as markers of endothelial dysfunction and tissue repair, respectively, by ELISA. *In vitro* studies were performed on human umbilical vein endothelial cells (HUVEC) exposed to healthy or uremic media. **Results:** The study included 26 hemodialysis (HD) patients (17 ± 3 months on dialysis, 52 ± 2 years, 38% men and 11% diabetic). Serum concentrations of CRP, IL-6, SDF-1 and IL-8 were 4.9 ± 4.8 mg/ml, 6.7 ± 8.1 pg/ml, 2625.9 ± 1288.6 pg/ml and 128.2 ± 206.2 pg/ml, respectively. There was a positive correlation between CRP and IL-6 ($\rho = 0.57$, $p < 0.005$) and between SDF-1 and IL-8 ($\rho = 0.45$, $p < 0.05$). *In vitro* results showed that after 6 hours treatment, SDF-1 expression by HUVEC treated with uremic media is lower compared to cells treated with healthy media ($p < 0.05$). After 12 hours of treatment there was an increase in IL-8 when HUVECs were exposed to uremic media ($p < 0.005$). **Conclusion:** We suggest that SDF-1 and IL-8 in HD patients can be used to measure the extent of damage and subsequent vascular activation in uremia.

Keywords: chemokine CXCL12; chemokines; endothelium, vascular; interleukin-8; renal insufficiency, chronic; uremia.

INTRODUCTION

In advanced stages of chronic kidney disease (CKD), most patients are affected by complications, most often correlated with cardiovascular disease (CVD) - including vascular calcification and endothelial dysfunction.[1,2] It is believed that the high concentration of circulating uremic toxins in this population may trigger systemic and vascular inflammatory responses, thereby inducing endothelial dysfunction,[3] a factor that is knowingly associated with CVD development and progression.

Studies led by our group showed that plasma markers of endothelial activation, such as monocyte chemoattractant protein-1 (MCP-1) and vascular adhesion molecule-1 (VCAM-1) are increased and closely associated with other markers of systemic inflammation in later CKD stages, such as CRP and IL-6. Furthermore, *in vitro* studies have shown that endothelial cell exposure to uremic environment increases MCP-1, interleukin-8 (IL-8) and VCAM-1 expression, suggesting a relationship between vascular injury, systemic inflammation and uremic toxicity.[4]

Endothelial cells play an important role in regulating vascular tone, homeostasis, blood pressure and vascular remodeling, and the endothelium's ability to synthesize and release nitric oxide (NO) is an important regulator of these physiological processes.[5] Bone-marrow derived endothelial progenitor cells (EPC) make up an endogenous endothelial repair system, protecting the endothelium from developing atherosclerosis. Evidence suggests that upon uremia there is a reduction in EPC

availability and function, leading to a loss in repair ability and endothelium regeneration, thus contributing to CVD development.[6]

The stromal cell-derived factor-1 (SDF-1) is a pleiotropic action chemokine expressed in various tissues such as the kidneys, bone marrow, liver, heart, thymus, spleen, skeletal and smooth muscle cells, endothelial cells and macrophages.[7-9] Originally, the SDF-1 action was related to the stimulation of T-lymphocytes, B-lymphocytes and monocytes.[10] However, it has been discovered that this chemokine also plays a key role in the pathophysiology of processes such as inflammation, angiogenesis, wound healing and platelet aggregation.[9,11-13]

Studies have shown that chemokines, such as SDF-1 and IL-8 possibly facilitate the migration of EPC to the injured endothelium. Indeed, SDF-1 has been described as an inducer of neovascularization, and together with IL-8 it is responsible for the recruitment of EPC to the ischemic tissue in CVD, such as acute myocardial infarction.[14,15] Recently, some studies have demonstrated that SDF-1 plasma levels are raised in CKD, correlating with decreased levels of EPCs.[16] These findings suggest a possible beneficial role of SDF-1 in the repair of endothelial injury.

From this overview, we hypothetically suggest that in CKD there is constant endothelium exposure to uremic toxins, with consequent vascular damage, which causes the release of chemokines involved in tissue signaling and repair. Thus, the aim of this study was to investigate the effects of uremic serum on *in vitro* expression of SDF-1 and IL-8 and conduct a clinical study involving patients with stage 5 CKD on hemodialysis (HD).

Methods

Patient selection

The patients of the study were selected from a population sample which included 104 chronic renal patients on HD treatment from a single center of renal replacement therapy in the city of Curitiba - PR. After applying the inclusion and exclusion criteria listed below, we finally selected 26 patients to participate in the study. All patients were on a chronic HD program and performed three HD sessions per week (3.5 to 4 hours per session) using polysulphone dialysis membranes and dialysate with a final sodium bicarbonate concentration of 32 mEq/L and 3.5 mEq/L of calcium. We excluded patients in peritoneal dialysis; patients who underwent HD sessions through a central venous catheter as vascular access; patients with infectious disease or severe chronic inflammation or malignancies; active liver disease; autoimmune diseases; use of immunosuppressive or anti-inflammatory agents in the last 3 months prior to enrollment in the study, and those who had had cardiovascular events (i.e. myocardial infarction, unstable angina, stroke or myocardial revascularization) 3 months before the study began.

All patients signed an informed consent agreeing to participate in the study and the protocol was approved by the Ethics Committee in Human Research of the Federal University of Paraná (CEP/SD: 974.079.10.07 registration). For comparison purposes, we used serum samples from healthy volunteers (n = 10) as control.

Clinical data collection

During patient recruitment, we collected clinical and demographic data through interviews and physical examinations performed on the initial assessment day and analysis of the medical records. We used the following data: age, gender, race, comorbidities, CKD primary etiology, time in dialysis, percentage of patients on statins, aspirin and vitamin D.

Collection of laboratory data

Blood samples were collected immediately before the first HD session of the week, centrifuged and stored at -80 °C. At baseline, we measured serum levels of total cholesterol, LDL and HDL cholesterol fractions; triglycerides; hemoglobin; albumin; calcium; phosphorus; parathyroid hormone (PTH), alkaline phosphatase and CRP in all patients.

In vivo experiments

Serum levels of systemic inflammation markers, SDF-1 and IL-8.

The C-reactive protein (CRP) test was performed by ultrasensitive automated immunoturbidimetry (ADVIA Chemistry System 1200, Siemens Healthcare, Deerfield,

Illinois, USA), with a detection range between 0.5 to 15 mg/L. IL-6 concentration was measured by the Enzyme Linked Immunosorbent Assay (ELISA) sandwich (R & D Systems, Minneapolis, USA) with a detection range between 0.5 to 15 mg/L. SDF-1 concentration was measured using ELISA (R & D Systems, Minneapolis, USA) with a detection range of 1.0 pg/ml to 47 pg/ml. The absorbance values were detected in a microplate reader (Tecan, North Carolina, USA) at 570 nm. IL-8 concentrations were measured using the in-house ELISA method (R & D Systems antibody, Minneapolis, USA), with a detection range between 31.25 to 2000 pg/ml. The absorbance values were detected in a microplate reader (Tecan, North Caroline, USA) at 570 nm. The intra and inter assay variation coefficients for IL-8 were 8.0% and 7.7%, respectively.

In vitro experiments

Extraction and characterization of human umbilical vein endothelial cells (HUVEC)

The umbilical cords were collected immediately after birth and processed within 24 hours. The endothelial cells were extracted and cultured according to Jaffe *et al.*[17] adapted.[4] Briefly, the umbilical cord was cannulated, washed with saline phosphate buffer (PBS) (Sigma-Aldrich, USA) and perfused with type II collagenase (Sigma-Aldrich, USA) at 0.3% in PBS for 7 minutes at 37 °C. The suspension was centrifuged and resuspended; and the pellet resuspended in medium 199 (Gibco, Grand Island, NY, USA) was supplemented with 2 mM glutamine (Gibco, Grand Island, NY, USA), fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) 10%, heparin 5,000 IU/ml (Sigma-Aldrich, USA), 0.5 mg/ml hydrocortisone (Sigma-Aldrich, USA), endothelial cell growth supplement at 15 g/ml (Sigma-Aldrich, USA); β-endothelial human cell growth factor 25 µg/ml (Sigma-Aldrich, USA); penicillin 10,000 IU/ml and streptomycin 50 µg/ml (Gibco, Grand Island, NY, USA). The HUVEC were grown to subconfluence in culture flasks of 25 cm^2 pre-treated with 1% gelatin (Sigma-Aldrich, USA) and incubated at 37 °C in an atmosphere of 5% CO_2. The cells were then used for experiments between passages 3 and 4, when they were trypsinized with 0.25% trypsin-EDTA (Sigma-Aldrich, USA) and further cultured in 96-well plates (10,000 cells/ml/well) pre-treated with 1% gelatin under the same conditions described above. The endothelial cell origin was confirmed by morphology and by immunocytochemistry with anti-CD31 monoclonal antibody (Dako Cytomation, Glostrup, Denmark).

Endothelial cells cultured with human serum

To prepare the uremic and the healthy mediums, we used sera from patients/individuals as pool. For this purpose, equal volumes of serum from all patients in the study (i.e., n = 26) formed a single uremic pool, and equal volumes of serum from healthy individuals (i.e., n = 10) formed a single control pool.

The HUVEC were initially cultured in 96-well plates until they reached subconfluence when they were maintained for a period of 12h in a suppression medium (199 medium plus 3% FBS) without growth factors. Subsequently, they were incubated in healthy medium (199 + 10% of the healthy pool) and/or uremic medium (199 medium + 10% uremic pool). Five experiments were performed in duplicate. The supernatants were collected at times: 0, 6 and 12h of culture and then stored at -80 °C until processing. For statistical analysis purposes we used the average of each duplicate.

Viability assay by the exclusion method with Trypan blue

The number of endothelial cells was determined by direct counting in a Neubauer chamber by the exclusion method with Trypan blue (Sigma-Aldrich, USA). After growing, the endothelial cells were treated with uremic and healthy media under the same conditions described above, trypsinized and resuspended in 1 ml of medium 199. Then, 10 µL of suspension were added to 10 µL of a 0.4% solution, and then we counted them in a Neubauer chamber with the aid of a light microscope (Nikon, Tokyo, Japan). The cells with dye uptake were deemed non-viable, and the number of viable cells was calculated by subtracting the number of non-viable cells from the total cell count.[18]

Viability assay by the 3-[4,5-dimetiazol-2yl]-2,5-ifeniltetrazolium bromide (MTT)

In this assay, the HUVEC (10^4 cells/ml/well) were cultured in a 96-well culture plate and subjected to the same treatments as described above with final treatment volume of 100 ml/well. The MTT (Sigma-Aldrich, USA) was solubilized in PBS at a

concentration of 5 mg/ml. Thereafter, the MTT solution was diluted 1:10 with 199 MEM medium (final concentration of 0.5 mg/ml) and added to the cells (100 ml/well). The plate was incubated for 4h at 37 °C. After this period, 100 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) were added to each well and the absorbance was measured at 570 nm.[19]

SDF-1 and IL-8 concentrations in the cell supernatant

The supernatant samples were collected at times: 0, 6 and 12h of incubation and stored at -80 °C until processing. The SDF-1 and IL-8 concentrations were measured by ELISA as described above.

Statistical analyzes

Statistical analyses were performed using the JMP statistical software for Windows version 7.0 (SAS Institute Inc., USA) and the SigmaStat version 3.5 software (Systat Software, Inc., Germany). Data was presented as mean ± mean standard error (MSE) or median (25^{th} and 75^{th} percentiles) as clinical and laboratory data for each parameter analyzed according to data symmetry or asymmetry. The results were analyzed by *t* test or one-way ANOVA for parametric data; and Mann-Whitney test for the nonparametric data. For multiple comparisons between the groups, we used the ANOVA on Rank's test followed by Dunnett's test. Correlation analyzes were performed using Spearman's test (ρ); and a $p \leq 0.05$ was considered significant.

Results

The main clinical and laboratory characteristics of the 26 patients enrolled in the study are depicted on Tables 1 and 2, respectively.

The mean age was 52 ± 2 years, and 38% were male. Hypertensive nephrosclerosis was the main cause of CKD, and all patients in the sample were hypertensive. Only 11% of the patients had *diabetes mellitus* as an associated disease. The patients were treated with statins, aspirin and anti-hypertensive drugs in 30%, 43% and 100% of cases, respectively.

Table 1 Main clinical characteristics of the population studied

Parameters	
Number of patients	26
Age (years)	52 ± 2
Gender (% men)	38
Ethnicity (% Caucasians)	81
Comorbidities (%)	
Diabetes mellitus	11
Cardiovascular diseases	15
Hypertension	100
Primary kidney disease (%)	
Hypertensive nephrosclerosis	30
Diabetic nephropathy	11
Chronic glomerulopathy	50
Other	9
Vitamin D (% used)	45
Statins (% used)	30
Aspirin (% used)	43
Anti-hypertensive agents (% used)	100
Time in dialysis (months)	17 ± 3

Values expressed in mean ± SD.

Table 2 Main laboratorial characteristics of the population studied

Parameters	
Cholesterol (mg/dL)	180 (108-248)
LDL Cholesterol (mg/dL)	106 (42-176)
HDL Cholesterol (mg/dL)	44 (21-80)
Triglycerides (mg/dL)	150 (73-240)
Hemoglobin (g/dL)	11.5 (10.8-12.0)
Albumin (g/dL)	3.9 (3.3-4.7)
Calcium (mg/dL)	9.0 (7.6-10.3)
Phosphorus (mg/dL)	6.7 (4.1-9.6)
PTH (pg/ml)	446 (11-1666)
Alkaline phosphatase (UI/L)	149 (65-602)
kt/V	1.5 (1.1-1.8)
CRP (mg/ml)	4.9 ± 4.8
IL-6 (pg/ml)	6.7 ± 8.1
IL-8 (pg/ml)	128.2 ± 206.2
SDF-1 (pg/ml)	2625.9 ± 1288.6

Values expressed as mean ± EPM or median (percentiles 25 to 75). LDL: Low density lipoprotein; HDL: High density lipoprotein; PTH: Parathyroid Hormone; CRP: C-Reactive Protein; IL-6: Interleukin 6; IL-8: Interleukin 8; SDF-1 Stromal cell-derived factor-1.

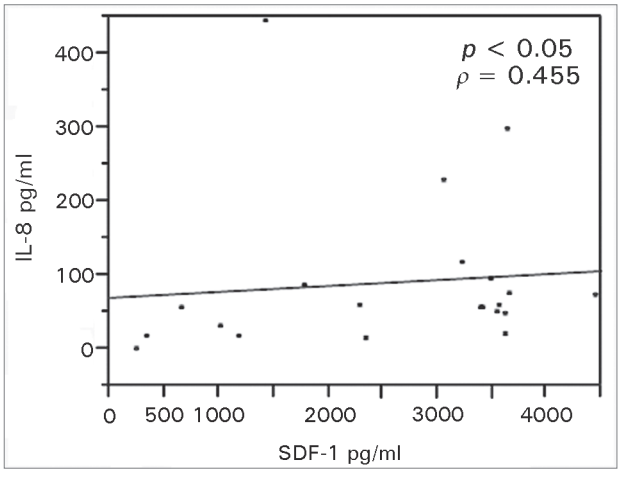
The median values found for total calcium, PTH, Kt/V, albumin and total cholesterol were within the reference range for patients on stage 5 CKD.

In vivo experiments

Markers of systemic inflammation and chemokines

The median serum concentrations of systemic inflammation markers, CRP and IL-6 were 4.9 ± 4.8 mg/ml and 6.7 ± 8.1 pg/ml, respectively. There was a positive correlation between CRP and IL-6 ($\rho = 0.57$, $p < 0.005$). SDF-1 and IL-8 concentrations were 2625.9 ± 1288.6 pg/ml and 128.2 ± 206.2 pg/ml, respectively. The correlation between the two chemokines is presented in Figure 1 ($\rho = 0.455$, $p < 0.05$). No significant differences were found between the median serum concentrations of SDF-1 and IL-8 considering the following variables: gender, ethnicity, primary renal disease and comorbidities. (data not shown). For the control group, SDF-1 and IL-8 serum concentrations were 1996.6 ± 259.7 pg/ml and 55.1 ± 33.9 pg/ml, respectively. There was no significant difference between serum levels of these two chemokines between the HD patients and the control group.

Figure 1. Correlation between IL-8 and SDF-1 serum concentrations in patients under hemodialysis treatment. IL-8: Interleukin 8; SDF-1: Stromal cell-derived factor-1.



In vitro experiments

Viability assay by the exclusion with Trypan blue method

The analysis of cell viability by the Trypan Blue exclusion method showed 95% viability for untreated HUVEC (control group, cells cultured with normal medium), 90% viability for HUVEC treated in healthy medium and 84% viability of cells treated with uremic medium. There was no significant difference between treatments when compared to the control group.

MTT viability assays

The MTT cell viability analysis showed no significant differences (*t*-test or one-way ANOVA) between normal culture medium, healthy medium or uremic medium applied to all treatments.

SDF-1 and IL-8 *in vitro* expression

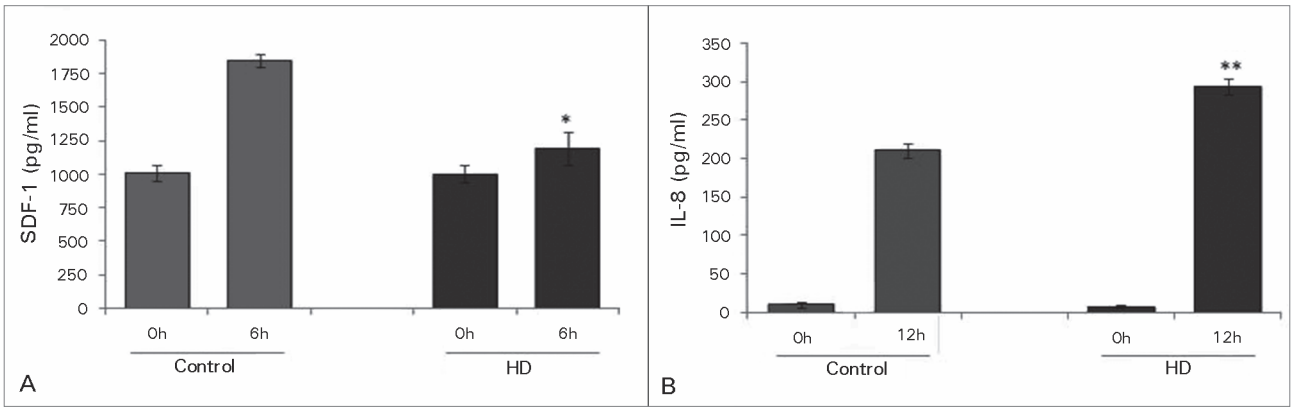
Figure 2 depicts the effect of uremic environment in *in vitro* expression of SDF-1 (A) and IL-8 (B) (pg/ml) on the HUVEC. After 6 hours of treatment, there is a lower expression of SDF-1 when HUVEC are treated with a uremic medium ($p < 0.05$), when compared to treatment with a healthy medium (*t*-test or one way ANOVA). After 12 hours of treatment, there is a significant increase in IL-8 when HUVEC are treated with uremic medium compared to the treatment with healthy medium ($p < 0.005$).

Discussion

During the last decade, several studies have demonstrated the action of uremic toxins as effector of endothelial dysfunction, contributing to CVD progression in patients with CKD.[20-22] Patients with CKD have an imbalance in endothelium-dependent vasodilation and increased circulating levels of endothelial dysfunction markers and oxidative stress. They also have an abnormal balance between cell damage caused by uremic toxicity and tissue repair (represented by decreased EPC migration), causing severe endothelial injury.[23] The main findings of this study show increased serum levels of IL-8 and SDF-1, markers of tissue injury and repair, respectively, in HD patients. Conversely, it was also shown *in vitro* that when endothelial cells are treated with uremic serum, they have decreased expression of SDF-1, but increased IL-8, suggesting a possible link between vascular activation and tissue repair in these patients.

The population included in this study comprised HD patients with chronic glomerulopathy, nephrosclerosis and diabetic nephropathy as the leading causes of CKD, and a high prevalence of CVD risk factors, such as hypertension. Regarding

Figure 2. A: *In vitro* SDF-1 (pg/ml) expression by HUVEC before and after (0 and 6h) treatment in uremic medium (HD). * $p < 0.05$ - Control 6h *vs.* HD 6h (test *t*); B: *In vitro* IL-8 (pg/ml) expression by HUVEC before and after (0 and 12h) treatment in uremic medium (HD). * $p < 0.005$ - Control 12h *vs.* HD 12h (test *t*).



the use of drugs, 45% were using vitamin D, 30% were on statins, 43% on aspirin and 100% were under antihypertensive drugs. No significant differences were observed between the study population and other previous studies conducted in HD patients,[24,25] except for the low prevalence of *diabetes mellitus* and dyslipidemia observed in our study. Serum markers of systemic inflammation, such as CRP and IL-6 were increased, and were also similar to those found in other studies, demonstrating that systemic inflammation is a common finding in HD patientS.[26,27] Yet, SDF-1 and IL-8 concentrations were also in agreement with other prior studies.[4,6,28,29]

The vascular endothelium has long been recognized as a complex endocrine organ, which regulates several physiological functions such as vascular tone, migration and growth of smooth muscle cells, vascular permeability to solutes and blood cells, homeostasis, among other functions.[30,31] Endothelial dysfunction can be broadly defined as a pro-inflammatory and pro-thrombotic state[32] and it is a frequent finding in CKD patients due to the constant exposure of the endothelium to uremic toxins, being regarded as a forerunner in the pathogenesis of atherosclerosis and obstructive arterial disease.[33,34] Thus, one can say that in these patients such conditions are closely related, but above all, mutually affected by one another.[35] In fact, in response to cellular injury, we have recently demonstrated *in vivo* and *in vitro*, that the endothelium exposure to uremic plasma dependent on uremia levels and time, increases the expression of MCP-1, soluble VCAM-1 (sVCAM-1) and IL-8, suggesting a link between vascular activation and uremic toxicity.[4] Also, some studies suggest that in CKD patients, there is an impaired angiogenic response due to the decreased production of mesenchymal stem cells mediated by SDF-1, vascular endothelial growth factor (VEGF) and VEGF receptor 1 (VEGFR1).[36]

SDF-1 is an important angiogenic factor released into the circulation in inflammatory processes, and it is responsible for the mobilization of EPC from the bone marrow into the circulation. The present study demonstrates that in HD patients, SDF-1 serum concentrations are increased when compared to healthy controls, positively correlating with IL-8. Such correlation occurs in parallel to the increase in CRP and IL-6 - systemic inflammation markers. In agreement with our data, Jie *et al.*,[6] - in studies involving patients with different degrees of CKD - suggested that vascular regeneration is poor even in the early stages of CKD, with increased levels of muscle progenitor cells vis-à-vis a decline in kidney function; this occurs concomitantly with an increase in SDF-1 plasma levels. Yet, studies show that after kidney transplantation, EPC levels are restored in parallel to the decline in SDF-1 levels, clearly demonstrating the role of uremia in cell injury and in regulating SDF-1 levels.[37]

In regards to vascular response activation of IL-8 production, our *in vitro* data confirm the *in vivo* results and found that in response to the uremic environment, endothelial cells increase the

levels of this chemokine, confirming uremia as the effector of cell injury. Conversely, *in vitro* results demonstrate that SDF-1 expression is reduced in endothelial cells upon exposure to uremic medium when compared to the cells exposed to the healthy medium, suggesting that uremia acts somewhat inhibiting the expression of this chemokine. In fact, Noh *et al.*[36] reported the uremic interference on the transcription of the CXCL12 (SDF-1) gene, inhibiting the synthesis of messenger RNA (mRNA); thereby decreasing the production of SDF-1. Also, Zaza *et al.*[38] observed in genomic studies of polymorphonuclear cells (PMN) from patients on HD that the CXCL12 gene expression is reduced, which could result in the accumulation of senescent PMN cells in the circulation.

Our *in vitro* results showed that after 6 hours of exposure to the uremic medium, the endothelial cells present with decreased levels of SDF-1 when compared to cells treated with the healthy medium. In part, this result can be explained because SDF-1 is also produced by other cells such as bone marrow cells, heart, liver, thymus, spleen, skeletal and smooth muscle cells, macrophages, kidneys cells, and endothelial cells acting in a pleiotropic manner;[7,8,39] this multiple production certainly reflects the serum levels of SDF-1 found in patients. Still, some studies show that after acute myocardial infarction, much of the subsequent angiogenic process is due to the joint action of SDF-1 and IL-8 in EPC recruitment to the site of injury.[14,15] These findings might explain the poor vascular adaptation found in patients with CKD after ischemic events.[40,41]

In conclusion, our *in vivo* results demonstrate that the action of uremia on HD patients may be associated with severe vascular damage, reflecting the increased circulating concentrations of IL-8 and SDF-1, suggesting a correlation between endothelial dysfunction and tissue repair. However, our study was limited to a small number of patients. We believe that studies with larger numbers of patients and additional *in vitro* tests are needed to evaluate possible causes for the *in vitro* reduction in SDF-1 levels.

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