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BIOMEDICAL SCIENCES

Assessment of the antioxidant status and markers of oxidative stress in patients with kidney failure: effects of a hemodialysis session

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Abstract: Data about the impacts of hemodialysis on antioxidant status and markers of oxidative stress are controversial, probably due to the use of different methodological approaches. The aim of this study was to assess the changes in the oxidative damage markers and antioxidant enzymes, and the serum antioxidant capacity by using *in vitro* model systems of free radical generation before and after one hemodialysis session. Blood samples were collected from 40 patients with kidney failure before and after hemodialysis. In pre- and post-hemodialysis serum samples, concentrations of biomarkers of oxidative damage and the activities of antioxidant enzymes were measured, as well as the *in vitro* antioxidant potential. The high concentrations of oxidative stress markers in serum of kidney failure patients were decreased after one hemodialysis session. In pre-hemodialysis, low activities of antioxidant enzymes were observed, including paraoxonase-1, however paraoxonase-1 activity was partially recovered after hemodialysis. Crocin bleaching and radical scavenging assays showed that serum antioxidant potential was decreased after hemodialysis. Although one hemodialysis session increased paraoxonase-1 activity and decreased oxidative stress markers, it caused a decrease in the serum antioxidant potential. Future research is needed to prospect strategies to mitigate the impacts of oxidative stress in the scenario of hemodialysis repetitions.

Key words: advanced glycation products, crocin bleaching assay, lipid peroxidation, paraoxonase, uremic solutes.

INTRODUCTION

Chronic kidney disease (CKD) has received global public health attention due to the high rates of prevalence, morbidity, and mortality. About 850 million people worldwide are estimated to have kidney disease, either acute kidney injury or CKD. Furthermore, by 2040, CKD is projected to be the 5th main cause of years of life lost globally (Francis et al. 2024). Hemodialysis (HD) is the commonest therapy for renal replacement in kidney failure patients, which contributes to the maintenance of homeostasis by allowing the removal of uremic toxins and fluid excess, diminishing the symptoms related to uremic syndrome (Murdeshwar & Anjum 2023). Approximately 4 million people worldwide are living on kidney replacement therapy (KRT), being HD the commonest KRT form, and accounting for 69% of all KRT (Bello et al. 2022). In Brazil, the estimated total number of CKD patients on dialysis in 2022 was approximately 153,831 individuals; from prevalent patients, 95.3% were on HD (Nerbass et al. 2024).

Although HD offers a temporary solution for kidney failure by replacing some excretory functions of the kidney, it does not efficiently diminish the rates of morbidity and mortality of patients with CKD (Qureshi et al. 2002). This scenario has been associated to the capacity of HD to act as an exacerbating factor of oxidative stress (Rysz et al. 2020), which can be attributed to some factors, including (*i*) activation of complement, platelets and polymorphonuclear cells triggered by blood exposure to dialytic membrane factors, with subsequent production of reactive oxygen species (ROS), and (*ii*) the loss of vitamins and other antioxidant molecules during HD (Liakopoulos et al. 2017). However, the literature reports contradictory results regarding changes in biomarkers of oxidative injury and antioxidant activity in individuals undergoing HD.

Oxidative stress plays a pivotal role in the progression of renal damage, leading to renal ischemia, glomeruli damage, cell death and apoptosis. Cardiovascular risk is also increased in kidney failure patients; oxidative stress during CKD causes endothelial dysfunction and reduces nitric oxide bioavailability, and has been associated with the formation of oxidized low density lipoprotein (ox-LDL), altogether these events participate in the pathogenesis of atherosclerosis (Rysz et al. 2020). Oxidative stress also contributes to the formation of advanced glycation end products (AGEs). Increased AGE concentrations are observed not only during hyperglycemia states; conditions associated with increased ROS concentrations are also accompanied by increases in AGEs, such as CKD (Stinghen et al. 2016). AGEs have been involved in the promotion of renal damage and acceleration of atherosclerosis in kidney failure patients (Noordzij et al. 2008).

In this study, we investigated the changes in the concentrations of markers related to biomolecules oxidation and advanced glycation, activities of antioxidant enzymes, as well as the antioxidant capacity by using *in vitro* model systems of free radical generation in serum of kidney failure patients before and immediately after one HD session.

MATERIALS AND METHODS

Study population

Ethics Statement: This study was approved (protocol number 76859617.4.0000.5426) by the Institutional Ethics Committee from the School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, SP, Brazil. Written informed consent was obtained from each patient after the explanation of the objective and the protocol of the study.

This study involved 80 subjects (18-60 years old), 40 blood donors (control group) and 40 patients with kidney failure undergoing HD (CKD group). Blood donors from control group (19 men and 21 women), non-smokers, without diseases or clinical outcomes, were selected from the Regional Hemonúcleo of Araraquara, São Paulo, Brazil. Patients from CKD group (22 men and 18 women) were recruited from the Regional Center of Hemodialysis, Araraquara, São Paulo, Brazil.

Inclusion criteria for the group having kidney failure patients experiencing HD over 3 months, 3 times per week, and at least 3-hour dialysis session duration. Patients underwent HD with bicarbonate solution in a capillary dialyzer with a high-flow polysulfone membrane. All patients had an arteriovenous fistula or vascular access, and the HD session was performed at blood flow rate were between 350 to 500 mL/min and dialysate flow rate between 500 to 800 mL/min. Patients performing peritoneal dialysis or patients having infectious diseases, hepatitis, neoplasms (benign or not), human immunodeficiency virus (HIV), hemolytic uremic

syndrome, tubulointerstitial nephritis, polycystic kidney disease and/or renal lithiasis were not included in the study.

Blood samples were collected using tubes without anticoagulants and centrifuged at 700 *g* for 10 minutes to obtain the serum, which was stored at -80° C until further analysis. Samples from kidney failure patients were obtained before the dialysis session (pre-HD) and immediately after the end of HD (post-HD), both were collected by vascular access.

Serum concentrations of creatinine, urea, uric acid, potassium, total protein, albumin, glucose, triglycerides, total cholesterol, and high density lipoprotein cholesterol (HDL-cholesterol) were performed in Vitros® 250 Chemistry Analyzer (Ortho Clinical Diagnostics, Johnson & Johnson Company). Low density lipoprotein cholesterol (LDL-cholesterol) and very low density lipoprotein cholesterol (VLDL-cholesterol) were estimated according to Friedewald et al. (1972).

Determination of biomarkers related to oxidative damage and antioxidant defenses

Products of lipid peroxidation (LPO) were determined in deproteinized serum samples via thiobarbituric acid reactive substances (TBARS) according to Kohn & Liversedge (1944). Thiobarbituric acid (TBA) reacted mainly with malondialdehyde, generating products (TBARS) whose concentrations were measured spectrofluorometrically (excitation and emission wavelengths of 510 and 553 nm, respectively). Standard curve consisted of 1,1,3,3-tetramethoxypropane (Sigma-Aldrich, USA). The results were expressed as micromoles per liter (µmol/L).

Fluorescent advanced glycation end products (AGEs) and pentosidine were estimated according to Pennacchi et al. (2015) with modifications. Supernatants from deproteinized serum samples were used to access the fluorescent AGEs (excitation and emission wavelengths of 370 and 440 nm, respectively) and pentosidine (excitation and emission wavelengths of 328 and 378 nm, respectively). The results were expressed as fluorescence arbitrary units (AU) per mg protein.

Protein carbonyl groups (PCO) were determined according to Levine et al. (1994). PCO reacts with 2,4-dinitrophenylhydrazine (DNPH), generating 2,4-dinitrophenylhydrazone that was monitored spectrophotometrically at 370 nm. PCO concentrations were calculated based on the molar extinction coefficient of the hydrazone (22,000 M^{-1} cm^{-1}). The results were expressed as µmol/L.

The activity of paraoxonase 1 (PON1) in serum samples was determined according to Assis et al. (2017) by the hydrolysis of paraoxon and release of *p*-nitrophenol, monitored spectrophotometrically at 405 nm. The results were expressed as mU/mg protein.

The activity of catalase (CAT) was measured as described by Johansson & Borg (1988) with modifications. In the presence of hydrogen peroxide (H_2O_2), CAT generates CAT compound I. Methanol is used as hydrogen donor for CAT compound I, and the formaldehyde produced was assessed spectrophotometrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald®, Sigma-Aldrich, USA) at 540 nm. The results were expressed as µmol/min/g protein.

The activity of glutathione peroxidase (GSH-Px) was determined according to Ferreira et al. (1999). GSH-Px catalyzes the oxidation of reduced glutathione (GSH) in the presence of H_2O_2, generating oxidized glutathione (GSSG). GSSG is reduced to GSH by glutathione reductase (GSH-Rd) with concomitant oxidation of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) to $NADP^+$. GSH-Px activity was determined by monitoring the NADPH oxidation

at 340 nm. The results were expressed as nmol of NADPH oxidized per min/g protein.

The concentrations of total sulfhydryl groups were measured as described by Sedlak & Lindsay (1968) with modifications. Sulfhydryl groups present in non-deproteinized samples reduce the 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) that was assessed spectrophotometrically at 412 nm. The results were expressed as µmol/g protein.

The crocin bleaching assay allows the *in vitro* analysis of the total antioxidant capacity (TAC). The method is based on the competition between crocin and antioxidant species to eliminate the peroxyl radical ($ROO^{\cdot}$). The assay was performed as described by Tubaro et al. (1998) and Assis et al. (2015) by monitoring the decrease of the crocin absorbance at 443 nm. The reaction was initiated by the addition of 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), which by thermolysis and in the presence of oxygen is decomposed to generate $ROO^{\cdot}$.

The decrease in the crocin bleaching rate in the presence of an antioxidant was analyzed by the kinetic equation of competition:

$$v_0/v = 1 + \text{slope} \cdot [A]/[C]$$

v_0, velocity in the absence of compound; v, velocity in the presence of compound; [A] concentration of the respective compound or samples; [C], crocin concentration (25 µmol/L). The slope of the regression line indicates the relative capacity of a compound to interact with $ROO^{\cdot}$.

The ABTS method allows the *in vitro* analysis of the antioxidant capacity, and was performed according to Re et al. (1999). The $ABTS^{\cdot+}$ radical cation was generated by the oxidation of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in the presence of potassium persulfate in the dark at room temperature for 16 hours. This solution was diluted in sodium phosphate buffer (pH 7.0) to obtain an absorbance of 0.750 ± 0.020 at 734 nm. The reaction mixture contained different concentrations of serum diluted in sodium phosphate buffer (pH 7.4) and solution containing $ABTS^{\cdot+}$ in sodium phosphate buffer (pH 7.0). The reaction was incubated for 15 minutes at room temperature in the dark and the absorbance was monitored at 734 nm. The results were expressed as effective concentration required to obtain 50% (EC_{50}) of antioxidant effect (percentage inhibition).

Statistical analysis

Data are expressed as mean ± standard error of mean. One-way analysis of variance followed by the Student-Newman-Keuls test was used to compare intergroup differences. Data were considered statistically significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, California, USA).

RESULTS

Impact of one HD session in the serum concentrations of biochemical markers, biomarkers of oxidative damage and antioxidant defenses of kidney failure patients

Patients with kidney failure before the HD session (pre-HD) showed significant increases in the serum concentrations of creatinine (14-fold), urea (4.5-fold), and uric acid (53%), as well as increased concentrations of potassium and glucose, when compared to the corresponding values in control group (Table I). One HD session was sufficient to decrease the concentrations of creatinine, urea and uric acid about 65%, 70% and 72%, respectively, and also the serum concentrations of potassium and glucose, in comparison with values of pre-HD samples (Table I).

Table I. Concentrations of biochemical parameters in the serum of kidney failure patients before (pre-HD) and after (post-HD) one HD session.

Parameters	Patients		
	Control (n = 40)	Pre-HD (n = 40)	Post-HD (n = 40)
Creatinine (mg/dL)	0.76 ± 0.036	10.51 ± 0.79$^{\#}$	3.63 ± 0.32$^{\#,\&}$
Urea (mg/dL)	28.4 ± 1.52	129.3 ± 7.16$^{\#}$	38.1 ± 2.62$^{\&}$
Uric acid (mg/dL)	4.64 ± 0.23	7.10 ± 0.28$^{\#}$	1.95 ± 0.09$^{\#,\&}$
Potassium (mmol/L)	4.50 ± 0.13	5.01 ± 0.15$^{\#}$	3.64 ± 0.15$^{\#,\&}$
Glucose (mg/dL)	95.1 ± 2.93	176.8 ± 15.98$^{\#}$	105.4 ± 6.80$^{\&}$
Total protein (g/dL)	7.52 ± 0.08	6.59 ± 0.11$^{\#}$	8.08 ± 0.23$^{\#,\&}$
Albumin (g/dL)	4.44 ± 0.10	3.67 ± 0.08$^{\#}$	4.62 ± 0.16$^{\&}$
Triglycerides (mg/dL)	137.3 ± 9.56	175.55 ± 19.78	162.87 ± 20.60
Total cholesterol (mg/dL)	183.5 ± 6.71	142.83 ± 7.59$^{\#}$	175.27 ± 10.02$^{\&}$
HDL-cholesterol (mg/dL)	58.1 ± 2.81	35.8 ± 1.90$^{\#}$	54.4 ± 2.82$^{\&}$
LDL-cholesterol (mg/dL)	99.2 ± 6.48	72.78 ± 5.68$^{\#}$	89.37 ± 7.71
VLDL-cholesterol (mg/dL)	27.46 ± 2.21	35.1 ± 3.96	32.57 ± 4.12

HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein.
Values are expressed as mean ± S.E.M., *n* = 40.
Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test. #: pre-HD *vs* Control; &: post-HD *vs* pre-HD.

Before HD, kidney failure patients had low serum concentrations of total protein, albumin, total cholesterol, HDL-cholesterol and LDL-cholesterol, in comparison to the corresponding values in control group. In contrast, the concentrations of these biomarkers were increased after one HD session. Triglycerides and VLDL-cholesterol concentrations did not differ among groups (Table I).

There were significant increases in the serum concentrations of TBARS (65%; Figure 1a), PCO (25%; Figure 1b), fluorescent AGEs (9-fold; Figure 1c) and pentosidine (22-fold; Figure 1d) in kidney failure patients before HD, in comparison with the control group. One HD session was able to reduce the serum concentrations of TBARS, fluorescent AGEs and pentosidine by 28%, 31% and 65%, respectively (Figures 1a, c and d), in comparison to the corresponding values in pre-HD, while the serum concentrations of PCO were slightly increased by 20% after HD (Figure 1b). It is important to mention that the correction of the PCO results by protein (total protein serum concentrations were increased by HD) implies equal PCO values between pre-HD and post-HD (PCO concentrations, nmol/mg protein: C = 0.180 ± 0.012; Pre-HD = 0.272 ± 0.028; Post-HD = 0.277 ± 0.016).

The activities of the antioxidant enzymes PON1, CAT and GSH-Px were 62%, 43% and 46% lower, respectively, in serum of kidney failure patients before HD than in control subjects (Figures 2a-c). The serum concentrations of thiol groups (representing a non-enzymatic antioxidant defense) were not changed in pre-HD patients in comparison to controls (Figure 2d).

In comparison with pre-HD, there were no differences in the activities of CAT in the serum of post-HD patients (Figure 2b), which remained low. On the other hand, one HD session had favorable effects on PON1, increasing by 75% its

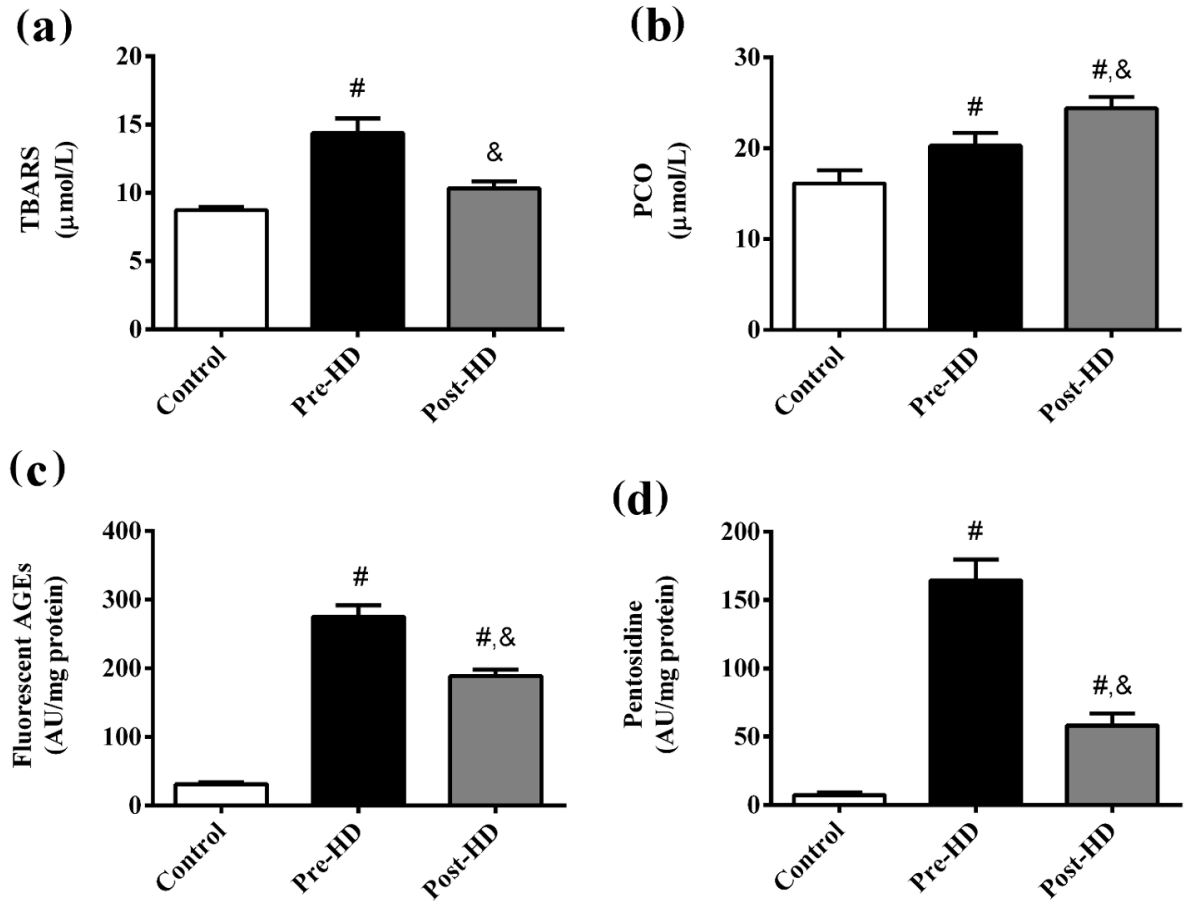

Figure 1. Biomarkers of oxidative damage in the serum of kidney failure patients before (pre-HD) and after (post-HD) one HD session. (a) TBARS; (b) PCO; (c) Fluorescent AGEs; (d) Pentosidine.
Values are expressed as mean ± S.E.M., *n* = 40. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test. #: pre-HD *vs* Control; &: post-HD *vs* pre-HD.

activity in comparison with pre-HD (Figure 2a). Conversely, one HD session caused a further decrease in the activity of GSH-Px (Figure 2c), but an increase in the serum concentrations of thiol groups (Figure 2d), when compared to the corresponding values in pre-HD patients.

Impact of one HD session in the antioxidant status in serum of kidney failure patients

To evaluate the contribution of some serum compounds in the antioxidant capacity of serum from pre-HD and post-HD, it was performed the analysis of the rate of the crocin bleaching inhibition in the presence of uric acid, ascorbic acid, Trolox, human albumin, and L-tyrosine. By comparing the values of the slope, the decreasing antioxidant capacity was: uric acid > ascorbic acid > Trolox > human albumin > L-tyrosine. Uric acid showed a great reduction in the rate of the crocin bleaching, suggesting that uric acid has high antioxidant capacity (Supplementary Material - Figure S1a-e).

The antioxidant capacity of compounds was also evaluated by determining the percentage of inhibition of the crocin bleaching and thus the EC_{50}, i.e., the effective concentration that inhibited 50% of the crocin bleaching (Table II). Uric acid was the most efficient compound

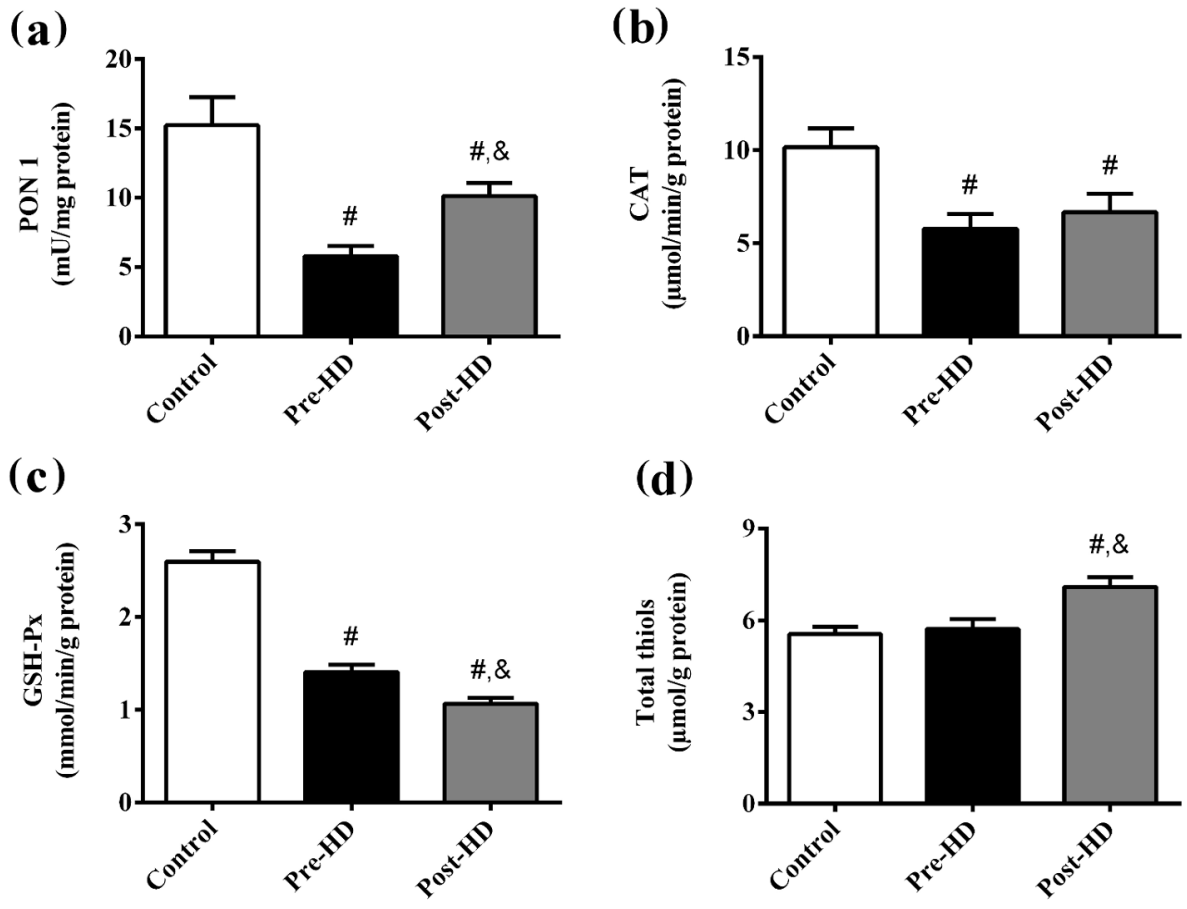

Figure 2. Endogenous antioxidant components in the serum of kidney failure patients before (pre-HD) and after (post-HD) one HD session. Activities of antioxidant enzymes (a) PON 1, (b) CAT and (c) GSH-Px and (d) concentrations of total thiol groups.
Values are expressed as mean ± S.E.M., *n* = 40. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test. #: pre-HD *vs* Control; &: post-HD *vs* pre-HD.

with the ability to protect the crocin oxidation, when compared to ascorbic acid, Trolox, human albumin, and L-tyrosine. The EC_{50} values found for the antioxidant compounds follow the same order of efficiency as the values of the slope. The lower the EC_{50} value, the more efficient its antioxidant action, while the greater the linear regression slope, the high antioxidant activity.

On the basis of these results, as well as knowing the antioxidant potential of these standard compounds, it was performed the analysis of the rate of crocin bleaching inhibition by serum samples obtained from kidney failure patients in the pre-HD and post-HD. Different serum volumes were used to obtain the percentage of inhibition of the crocin bleaching. Since the blood serum is a complex sample that contains a mixture of antioxidants at different concentrations, the antioxidant capacities of serum samples from control and kidney failure patients were expressed in terms of "EV_{50}" (µL), i.e., the volume of serum that inhibited by 50% the crocin bleaching (Figure 3).

Kidney failure patients in the pre-HD had low "EV_{50}", which represents a great antioxidant capacity, in comparison with the "EV_{50}" of the

Table II. Comparison between EC_{50} (µM) and linear regression slope for uric acid, ascorbic acid, Trolox, human albumin, and L-tyrosine in the crocin bleaching assay.

Compounds	EC_{50} (µM)*	Linear regression slope**
Uric acid (µM, expressed in EC_{50})	8.17 ± 0.065	3.3045
Ascorbic acid (µM, expressed in EC_{50})	9.45 ± 0.430	2.6145
Trolox (µM, expressed in EC_{50})	10.41 ± 0.075	2.0131
Human albumin (µM, expressed in EC_{50})	52.77 ± 0.995 [a,b,c]	0.5542
L-tyrosine (µM, expressed in EC_{50})	*** *Nihil*	0.0130

*** EC_{50} values in decreasing order of efficiency, obtained through the equation:**
**** Linear regression slope values in decreasing order of efficiency.**
***** No EC_{50} value was observed up to 1250 µM.**
a: different compared to Uric acid; b: different compared to Ascorbic acid; c: different compared to Trolox.

control group and kidney failure patients in the post-HD (Table III). The minor values of "EV_{50}" from pre-HD indicated that, with a low volume of serum, the "EV_{50}" was reached for the crocin assay. It was also observed that the "EV_{50}" values found in serum samples follow the same order of efficiency as the values of the slope.

Figure 4 shows the relation between the ratio of velocities *versus* the ratio of fraction of serum volumes and crocin. The curves were plotted with a range of velocity ratio values (v_0/v) and the mean tangent of each group (Table III). The values of the ratio between the concentrations of the antioxidants present in the serum by the concentration of crocin were replaced by the respective fractions of serum volumes and the fraction of crocin volume, represented by A/C, allowing the analysis of the kinetic competition. This procedure was also applied to the Trolox (for which it is known its concentration, as well as the volume fraction used in the assay), and it was observed the same behavior when using concentration analysis.

This analysis showed a linear behavior within each group of subjects (control group and patients with kidney failure before and after the HD session). Since these curves were plotted from the averages of the slope values presented in the Table III, it is also clear that pre-HD patients showed kinetics consistent with the slope, thus demonstrating a great antioxidant capacity in the serum, when compared with both the control group and the post-HD patients (Figure 4).

Data from the compounds studied in the $ABTS^{\cdot+}$ scavenging assay were used to calculate the EC_{50}, i.e., the effective concentration of the compound capable of capturing 50% of the $ABTS^{\cdot+}$ radicals present in the reaction (Figure S2). L-tyrosine proved to be more efficient in capturing the $ABTS^{\cdot+}$, showing EC_{50} of 2.69 µM, followed by Trolox (15.06 µM), uric acid (15.78 µM), ascorbic acid (18.30 µM), and human albumin (19.08 µM).

In Figure 5, the percentage of the $ABTS^{\cdot+}$ inhibition by the volume fraction (different dilutions of serum in the reaction) of serum samples from one subject of control group is shown in a representative way. It was obtained the "EV_{50}", i.e., the sample volume needed to inhibit 50% of $ABTS^{\cdot+}$. The linear region of the curve (inserted graph) was analyzed. It was noted that, the more diluted the serum, the greater is the absorbance of the radical and, therefore, the less capacity of the sample (serum) to capture $ABTS^{\cdot+}$.

The antioxidant capacity of a complex sample can be expressed in terms of the antioxidant

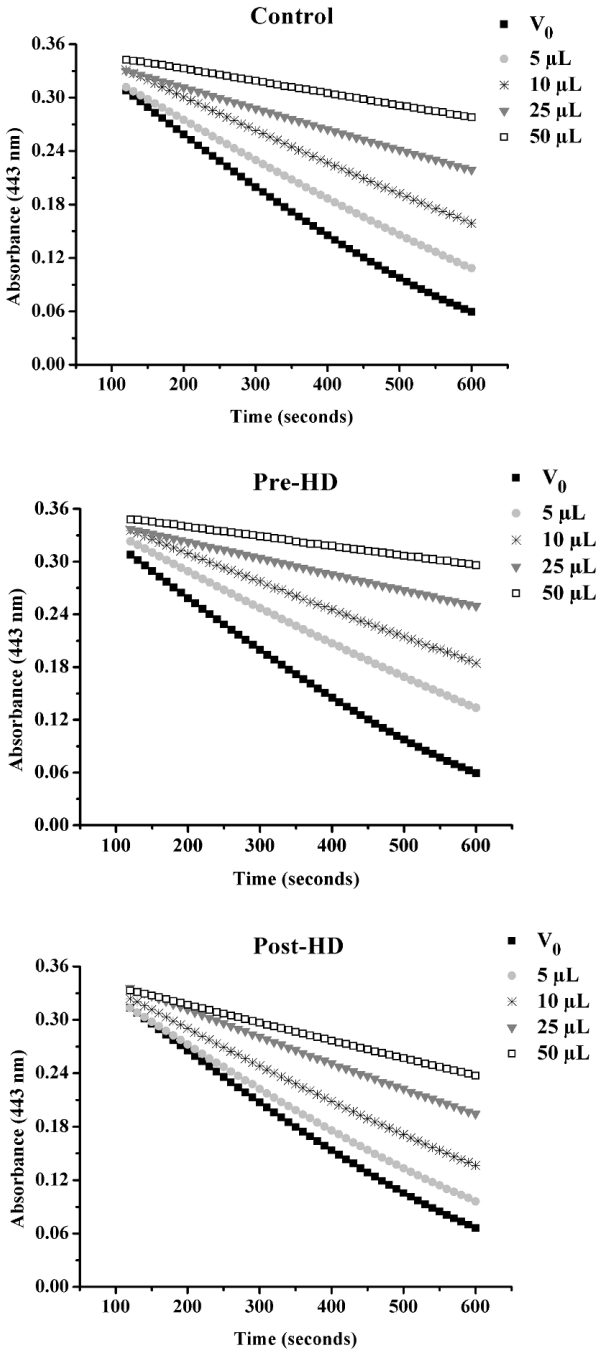

Figure 3. Representation of the velocity ratios plotted against different volumes of serum from subjects of the control group and patients with kidney failure in the pre-HD and post-HD for the crocin bleaching assay. The graphics represents the decrease in the absorbance of crocin at 443 nm: (v_0) velocity in the absence of serum and (v) velocity in the presence of different volumes of serum (5, 10, 25 and 50 µL).

capacity of standard compounds. Trolox is commonly used as a standard antioxidant compound, and the results are expressed as Trolox Equivalent Antioxidant Capacity (TEAC). For human serum, the results can also be expressed as biomolecule equivalent (uric acid or human albumin).

Table III shows the "EV_{50}" of the serum volume fraction of subjects from controls or kidney failure patients before and after HD. There were no differences in the "EV_{50}" values between control group and pre-HD patients; however, after the HD session, there was an increase (14%) in the "EV_{50}", when compared to pre-HD. Serum samples from kidney failure patients in the pre-HD had a great equivalence to Trolox, uric acid and human albumin, when compared to the serum of control group (Table III). After HD, there was a decrease in the equivalence to Trolox, uric acid and human albumin, when compared to serum samples before HD.

The equivalences to Trolox, uric acid and human albumin showed the same response profile, i.e., serum samples from pre-HD had great equivalence to standard compounds, when compared to the control group and to the post-HD (Table III). The equivalence to uric acid is correspondent to the uric acid concentrations (Table I) in serum samples of the groups studied. However, the serum albumin concentrations and the equivalence to human albumin did not follow this same profile; there was a significant increase in the albumin concentrations (Table I) in serum samples from post-HD patients and a decrease in the equivalence to human albumin in the post-HD serum samples.

DISCUSSION

Accumulation of biomarkers related to oxidative damage has been observed in uremic conditions. Therefore, CKD is known to promote

Table III. Mean values of "EV_{50}" (µL) and linear regression slope (crocin bleaching assay) and mean values of "EV_{50}" of the fraction of the serum volume and equivalents to Trolox, uric acid and human albumin (ABTS method) of the control group and CKD patients before (pre-HD) and after (post-HD) a HD section.

Crocin Bleaching Assay				
Samples	"EV_{50}" (µL)*	Linear regression slope		
Control	29.05 ± 1.48	0.3389		
Pre-HD	15.76 ± 1.38$^{\#}$	0.5222		
Post-HD	38.07 ± 1.92$^{\#,\&}$	0.2243		
ABTS Method				
Samples	"EV_{50}" (µL)	Equivalents (µM/µL) of serum samples		
		Trolox	Uric acid	Human albumin
Control	0.00391 ± 0.00010	12.82 ± 0.31	13.46 ± 0.32	16.48 ± 0.40
Pre-HD	0.00367 ± 0.00010	13.98 ± 0.37$^{\#}$	14.67 ± 0.39$^{\#}$	17.97 ± 0.48$^{\#}$
Post-HD	0.00418 ± 0.00017$^{\&}$	12.22 ± 0.44$^{\#,\&}$	12.83 ± 0.46$^{\#,\&}$	15.71 ± 0.56$^{\#,\&}$

Values are expressed as mean ± S.E.M., *n* = 40.
***EV_{50} values obtained through the equation: (for the crocin bleaching assay).**
Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test. #: pre-HD *vs* Control; &: post-HD *vs* pre-HD.

oxidative stress by a combination of increased production of oxidant molecules and decreased concentrations of antioxidant enzymes (Ratliff et al. 2016). Increased concentrations of oxidant molecules contribute to the progression of CKD to advanced stages, as well as favor the development of diabetic nephropathy, severe inflammation, and endothelial dysfunction (Harlacher et al. 2022). According to our findings, there were significant increases in the serum concentrations of TBARS in kidney failure patients in the pre-HD. Several conditions can contribute to the LPO promotion in CKD, such as comorbidities (diabetes mellitus, arterial hypertension), accumulation of uremic toxins, iron excess acting as pro-oxidant (iron supplementation is recommended for kidney failure patients due to anemia), and the HD itself promoting oxidative stress. After HD, the TBARS serum concentrations were decreased in kidney failure patients. It has been suggested that some LPO products can diffuse through the dialysis membranes and thus be removed by HD (Gerardi et al. 2002). On the other hand, it was observed that HD appears not to be fully effective in normalizing TBARS concentrations in kidney failure patients, due to the fact that bound LPO products (in proteins, nucleic acids, and lipoproteins) are difficult to be removed by HD (De Vecchi et al. 2009).

Protein carbonyls have been noticed to be increased in kidney failure patients (Miyata et al. 2000). In addition to the direct deleterious impacts of ROS in proteins of kidney failure patients, an increased generation of carbonyl groups in proteins may be also a consequence of the increased concentrations of AGEs and LPO products (Dursun et al. 2005). In agreement with this, our study demonstrated that the serum concentrations of TBARS and AGEs are significantly increased in kidney failure patients (pre-HD) in parallel to the increased concentrations of PCO. Furthermore, data about the impacts of HD on PCO concentrations often indicate that HD causes a further increase in the PCO in comparison with pre-HD. The increase in

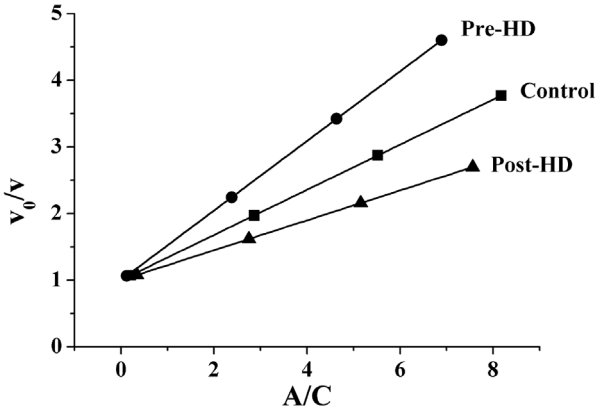

Figure 4. Curves representing the range of speed ratio values (v_0/v) and the average tangent of each group, thus obtaining the respective serum volume fractions. A, serum volume fraction; C, crocin fraction; v0, absence of serum; v, presence of serum (in crocin bleaching assays). A/C represents the ratio between serum volume fractions and crocin volume fraction.

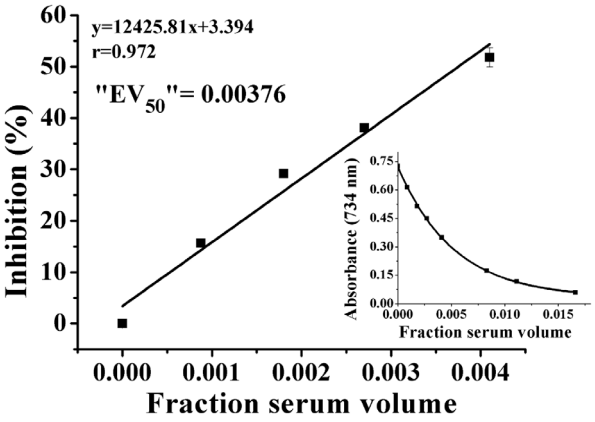

Figure 5. Representation of the $ABTS^{\bullet+}$ scavenging capacity ("EV_{50}") of different serum dilutions from one subject of the control group.
The inserted graph represents the mean of the absorbances (in 734 nm) of $ABTS^{\bullet+}$ in different fractions of the serum volume.

PCO concentration due to HD has been attributed to some factors, such as incomplete removal of uremic solutes, bioincompatibility of dialytic membranes, and the loss of low molecular weight antioxidants due to HD removal (Colombo et al. 2018). In our study, it was found an additional increase in the serum PCO concentrations in kidney failure patients after HD in comparison with pre-HD, which is in agreement with previous findings (Colombo et al. 2018, Dursun et al. 2005). However, in addition to attribute this increase in the PCO concentrations only to the exacerbation of oxidative stress caused by HD, it must be considered that this increase is also related to hemoconcentration. This hypothesis is reinforced considering the following: (*i*) the correction of the PCO results by total protein, whose concentrations were increased by HD, implies equal PCO values between pre-HD and post-HD; (*ii*) markers of oxidative damage that have a non-protein bound fraction (TBARS, AGEs) had their concentrations decreased after HD.

Serum AGE concentrations are often increased in kidney failure patients, even in the absence of chronic hyperglycemia. The accumulation of AGEs in CKD results from decreased glomerular filtration rate, as well as inflammation and oxidative stress (Stinghen et al. 2016). AGEs can exert deleterious effects via interaction with RAGE receptors, interfering with various cellular responses and exacerbating ROS generation, and causing endothelial dysfunction. The detrimental events related to AGEs contribute to the increased prevalence of cardiovascular diseases and to the progression of atherosclerosis in kidney failure patients (Linden et al. 2008). According to our findings, one HD session was able to reduce the serum AGE concentrations in kidney failure patients. It has been observed that one HD session was capable of reduce the AGE concentrations, but only those of low molecular weights, and the efficiency of AGE removal can be improved by increasing the frequency or the duration of the HD session (Stinghen et al. 2016).

According to our results, the activities of the antioxidant enzymes CAT, GSH-Px and PON1 were significantly low in serum from kidney failure patients. It has been proposed that the decrease in the CAT activity is a consequence

of the detrimental impacts of $O_2^{\cdot-}$ and H_2O_2 in the enzyme structure, contributing to the loss of activity and thereby resulting in increased LPO (Aziz et al. 2016). Perhaps for this reason, one HD session was not enough to recover CAT activity. The selenium-dependent enzyme GSH-Px3 has catalytic potential to detoxify H_2O_2, lipid peroxides, and organic hydroperoxides. It is synthesized by kidneys, secreted into the bloodstream and is found to be associated with HDL (Brites et al. 2017). Therefore, it can be suggested that the decrease in the GSH-Px activity in kidney failure patients may be a consequence of a decrease in its expression by renal cells due to kidney dysfunction. Post-translational modifications of GSH-Px including glycation, oxidation and carbamylation have been also associated with impairments in its activity (Roxborough et al. 1999). It has been shown that selenium concentrations are decreased in patients under HD (Fujishima et al. 2011), which helps to explain the slight additional decrease in the GSH-Px activity after one HD session. PON has the ability to hydrolyze lipid peroxides; PON1 and PON3 isoforms are associated with HDL. Both the low PON1 concentrations and its inactivation by AGEs and uremic toxins have been proposed to explain the decrease in the PON1 activity in kidney failure patients (Gugliucci et al. 2007). In fact, the recovery in the PON1 activity after dialysis reinforces that uremic toxins and AGEs may play a mechanistic role in enzyme inactivation (Gugliucci et al. 2007) which helps to explain our findings about the partially restoration of serum PON1 activity in kidney failure patients after HD.

Organic compounds having thiol groups act as main components in endogenous antioxidant defenses. Most thiol groups in plasma are found in albumin, and in a lesser extend in low molecular weight compounds, including free cysteine, glutathione, and homocysteine. Although we did not find differences in the serum thiol concentrations of kidney failure patients (before HD) when compared to controls, one HD session was capable to increase their concentrations, as previously observed (Ayar et al. 2018). It can be suggested that the hemoconcentration of proteins, mainly albumin, is responsible for the increase in the thiol groups after HD; thiol groups in albumin represent the largest fraction of plasma thiol (Turell et al. 2013).

By using an *in vitro* model system of LPO, Assis et al. (2015) evaluated the total antioxidant capacity (TAC) of some uremic solutes; in order of efficiency, uric acid, *p*-cresol, phenol and L-tyrosine presented great antioxidant potential. Our findings showed that serum of kidney failure patients, in the pre-HD, have great TAC, since a low serum volume was necessary to inhibit by 50% the crocin oxidation by $ROO^{\cdot}$. High TAC values in kidney failure patients from pre-HD can be attributed to the presence of molecules with antioxidant potential, whose serum concentrations are increased before HD, including uric acid. Malliaraki et al. (2003) also observed that serum of kidney failure patients in the pre-HD have great antioxidant capacity due to increased concentrations of endogenous metabolites (such as uric acid). After HD, we observed a significantly reduction of serum uric acid concentrations, and consequently a decrease in TAC values. The loss of low weight antioxidant molecules due to HD can be compensated by other molecules, although less efficiently, including albumin. Albumin binds to metal ions (copper, iron) that are very potent to generate ROS, as well as is able to scavenge hydroxyl and peroxynitrite radicals (Roche et al. 2008).

In our present study, it is also possible to compare the findings about the serum concentrations of TBARS (LPO biomarkers) and data from the *in vitro* model system of

LPO (crocin bleaching). Kidney failure patients in the pre-HD showed significant increases in the serum TBARS concentrations in parallel to a great antioxidant capacity according to the crocin bleaching assay. It can be suggested that the increase in the TBARS concentrations is a consequence, at least partially, of lipid oxidative damages that are occurring in various tissues, while the crocin bleaching assay allows to analyze the ability of molecules present in the serum to protect the crocin against oxidation by $ROO^{\cdot}$. Uric acid and Trolox are commonly used as standard antioxidants. Uric acid has a great ability to eliminate carbon-centered radical and $ROO^{\cdot}$ in hydrophilic aqueous medium. Trolox is an antioxidant molecule analog of vitamin E; it has a phenolic hydroxyl group involved in the capture of free radicals. Human plasma is highly resistant to oxidation, due to the presence of molecules having antioxidant properties, including uric acid, ascorbic acid, vitamin E, thiol antioxidants, antioxidant enzymes and other proteins (Kurutas 2016). In fact, uric acid confers about 60% of the total plasma antioxidant capacity in humans. Proteins (mainly albumin) contribute to the great antioxidant capacity in plasma mainly due to the presence of thiol groups from cysteine residues. However, other amino acid residues can also contribute to the protection against damage oxidative, including tyrosine since it contains a phenolic hydroxyl group that donates hydrogen and thus is able to react with oxygen or nitrogen in termination reactions, preventing radical generation. Our results showed that, among control, pre-HD and post-HD groups, the serum from pre-HD patients had the highest TAC, in both the crocin bleaching assay and $ABTS^{\cdot+}$ capture assay. Ruskovska et al. (2014) also observed that patients before HD had great TAC in comparison to control subjects, and that after one HD session, there was a decrease in TAC when compared to pre-HD, suggesting that the abundance of small antioxidant molecules in pre-HD serum samples is contributing to the antioxidant capacity.

In conclusion, the present study provides evidence for the improvements of one HD session in some oxidative stress parameters in serum of kidney failure patients, including the low concentrations of oxidative damage biomarkers and the partial recovering in the activity of the antioxidant enzyme PON1. However, by using *in vitro* model systems of free radical generation, it was possible to observe that one HD session also caused a significant decrease in the serum antioxidant potential in comparison with pre-HD. Paradoxically, until the next HD session, the recovery of serum antioxidant potential may occur due to accumulation of uremic toxins with radical scavenging capacity. For this reason, as quoted by Liakopoulos and collaborators (2017), "oxidative stress should be incorporated in a "uremic milieu" abnormality approach and might constitute a novel but quite important therapeutic target in chronic HD patients (...) assessing oxidative stress status in HD patients is a matter under discussion and probably in the light of more solid evidence could be incorporated in future routine clinical practice or even guidelines". Therefore, future studies are needed to prospect efficient strategies to mitigate the impacts of oxidative stress in the scenario of HD repetitions.

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SUPPLEMENTARY MATERIAL

Figures S1, S2.

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