

Evaluation of endothelial adaptation and parameters of oxidative stress in patients after hemodialysis sessions

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ARTICLE INFO

Article history:

Received on: 30/05/2013

Revised on: 15/06/2013

Accepted on: 05/07/2013

Available online: 31/10/2013

Key words:

Free radicals, renal insufficiency; endothelium; Nitric oxide; IMA.

ABSTRACT

This work was focused on the evaluation of parameters of oxidative stress (lipid peroxidation, total thiol groups, non-protein thiol groups, polyphenols and ascorbate) and endothelial adaptation by determining the nitric oxide (NO), high-sensitive C-reactive protein (HS-CRP) and ischemia-modified albumin (IMA) of patients with chronic renal insufficiency submitted to hemodialysis. It was seen a depletion of glutathione, polyphenols and reduced ascorbate, combined with the increase of oxidized ascorbate and lipid peroxidation stress. In addition, was verified the increase of levels of IMA in the plasma. NO showed an evident endothelial protective role due to reduction of levels of HS-CRP, however, did not contribute to increase the levels of IMA. Based on the results, it can be concluded that the patients evaluated in this study presented a clinical situation of oxidative stress. A new study focusing on the prevention of oxidative stress using different experimental models with antioxidants is valuable to improve the life quality of these patients.

INTRODUCTION

Nowadays, renal insufficiency is an important public health problem, affecting 40 millions of people in Brazil and in the world. Actually in Brazil, about of 60.000 persons 41 are submitted to dialysis, being that 25.000 began the treatment each year (SBN, 2012). However, there is an estimative that at least 150.000 persons should be submitted to the same procedure (SBN, 2012). The extracorporeal circulation during hemodialysis is identified by the body as an aggressive agent, promoting a series of adaptations. One of the most important reactions of the body to the extracorporeal circulation is the systemic inflammatory response (Clermont *et al.*, 2000). The reintroduction of the oxygenated blood also could promote some lesions on tissues, with consequent releasing of oxygen free radicals. These radicals are generated during the ischemic reperfusion causing the oxidative stress that promotes damage to the affected tissues or organs (Modlinger *et al.*, 2004). Cardiovascular alterations are frequent in patients that are undergoing to the hemodialysis, being responsible by about 50% of the deaths in this group of patients (Shlipak *et al.*, 2005).

The endothelium is a monolayer of cells that act as passive barrier between the plasma and the extracellular liquid, which is a great producer of nitric oxide (NO) (Witko-Sarsat *et al.*, 1996; Pearson *et al.*, 2003). NO is biosynthesized endogenously from L-arginine, oxygen and NADPH by various nitric oxide synthase (NOS) enzymes. Nitric oxide (NO) contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium. Nitric oxide is also generated by phagocytes (monocytes, macrophages, and neutrophils) as part of the human immune response. In this way the immune system may regulate the armamentarium of phagocytes that play a role in inflammation and immune responses. Nitric oxide secreted as an immune response is as free radicals and is toxic to bacteria Witko-Sarsat *et al.*, 1996). The high-sensitive C-reactive protein (HS-CRP) is a serum marker for inflammation, which is easily and inexpensively measured and assays have recently been developed that provide very high sensitivity. Numerous epidemiologic studies have consistently shown that high-sensitivity C-reactive protein (HS-CRP) levels provide a strong and independent indication of risk of future heart attacks, ischemic stroke (due to obstruction, not bleeding), and peripheral arterial disease, even among individuals who are thought to be free of

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vascular disease (Pearson *et al.*, 2003; Ridker *et al.*, 2007). The ischemia-modified albumin (IMA) has been pointed out as an excellent marker of cardiac ischemia. However, some studies are reporting that increasing IMA is a positive indicative of mortality in patients with chronic renal disease in final stage (Sinha *et al.*, 2003; Quiles *et al.*, 2003). Although there are endogenous defense mechanisms against free radicals, whenever the activity of cellular antioxidant systems decrease or when the amount of free radicals exceeds the capacity of neutralization it is verified the oxidative damage to cells, leading to several pathological conditions such as premature aging, lipid peroxidation and damage to cell membranes. In addition, the high levels of lipid and protein peroxidation as well as decreasing the antioxidant activity have been verified in uremic patients, supporting the hypothesis that these individuals have their defense mechanisms decreased against the oxidative stress (Bandyopadhyay *et al.*, 1999).

The presence of endothelial damage is a consistent finding in patients with chronic renal insufficiency and has been recognized as a new risk factor to coronary disease. Several evidences have been suggested that chronic inflammation is crucial to the development and progression of the atherosclerosis in patients with chronic renal insufficiency submitted to hemodialysis (Stenvik).

Some authors report that the oxidative stress has direct consequence on induction of many pathological processes as cancer, autoimmune diseases, heart defects and lung diseases (Mayr *et al.*, 2006). However, a better understanding of the oxidative effects on patients undergoing hemodialysis is essential, what requires further biochemical studies. In addition, there are a lack of studies that establish evident relationship between oxidative stress and endothelial adaptation.

In this sense, the main objective of this work was evaluated the production of inductors of oxidative stress of patients after hemodialysis sessions. In addition, it was evaluated the endothelial adaptation by determining the nitric oxide, high-sensitive C reactive protein and ischemia-modified albumin.

MATERIALS AND METHODS

Materials

Materials used for biochemical analysis obtained from Merck (Rio de Janeiro, RJ, Brazil) were: glacial acetic acid, sulfuric acid, perchloric acid, ascorbic acid, mercuric chloride, thiourea, dinitrophenylhydrazine and Folin-Ciocalteu. The 5,5'-dithiobis (2-nitrobenzoic acid), thiobarbituric acid and DL-dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). And trichloroacetic acid, copper sulfate and sodium chloride from Reagen (Rio de Janeiro, RJ, Brazil).

Patients

We investigated 25 patients with chronic renal insufficiency submitted to hemodialysis from highly complex hospital in southern Brazil (S27° 38.1799', W052° 16.9966'). The age of patients ranged from 22 to 80 years old. All patients gave

written informed consent, and this study protocol was approved by the institutional ethics committee (number 126/TCH/09).

Blood samples were collected by central venous catheter puncture. Four milliliters of collected blood samples were transferred into tubes containing 2 mg/mL of ethylene diamine tetraacetic acid (EDTA) and 5 mL into tubes containing no anticoagulant. Samples were routinely centrifuged within 1 hr of collection for 15 min at 1.500 rpm, and aliquots of serum samples were stored at -18°C for a maximum of 2 weeks before the analysis.

Biochemical Analysis

Thiol group (mM) measurements in plasma were carried out using the Ellman reagent. Thiols react with this reagent, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB-), which ionizes to the NTB2- dianion in water at neutral and alkaline pH.

This NTB2- ion has a yellow color and was quantified in a spectrophotometer by measuring the absorbance at 412 nm, using an extinction coefficient of 14,150 M⁻¹ cm⁻¹ (Ellman, 1959). The measurement of phenolic compounds in the plasma was carried out by Folin Ciocalteu Reagent (FCR) method measuring the absorbance at 765 nm according to Waterman and Mole (1994). The results were expressed as Gallic Acid Equivalent (mg.L⁻¹ GAE).

The lipid peroxidation was assessed indirectly by quantifying the malondialdehyde (MDA) by reacting the lipid peroxides with thiobarbituric acid (TBA), according to method reported by Ohkawa *et al.*, (1979).

Total plasma vitamin C concentration (mg.L⁻¹) was measured by spectrophotometer According to the method proposed by Jacques-Silva *et al.*, (2001). Ascorbic acid of the sample was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. In addition, two color reagents were used: the first contained 4.5 mg.mL⁻¹ dinitrophenyl hydrazine and 0.6 mg.mL⁻¹ thiourea in a 9 mol.L⁻¹ H₂SO₄ solution in the medium (1 ml), and the second was equal to the first plus 0.075 mg.mL⁻¹ CuSO₄. An aliquot of the sample in a final volume of 1 ml of the solution was incubated for 3 hr at 38°C, then 1 ml H₂SO₄ 65% (v.v⁻¹) was added to the medium. Samples from the same individual were used for two distinct determinations, i.e., initial and total dehydroascorbic acid with the first and second reagent, respectively. The difference between these values was considered to be the amount of ascorbic acid in the sample.

High-sensitive C-reactive protein was determined with a Biotechnical Reactive C Protein Turbidimetric Kit with specific high-sensitivity methodology for Cardiology, with linearity to 0.1 up 15mg.L⁻¹ (Biotécnica®, Minas Gerais, Brazil). The ischemia-modified albumin was assessed according to the method proposed by Bhagavan *et al.*, (2003). The nitric oxide was determined by means of the Griess assay, using a standard curve using sodium nitrite as standard, conform described by Bracht and Ishii-Iwamoto (2003).

Statistical Analysis

Test results were compiled for a worksheet of the spreadsheet and the results were transferred to SPSS 13.0 for Windows. Data were tested for normality by Kolmogorof-Smirnoff analysis, followed by analysis of Student's t test for paired samples, with a minimum level of significance of $p \leq 0.05$. Data were expressed as mean \pm standard error.

RESULTS

Figure 1 shows the results of total glutathione, where is verified a statistically significant increase ($2.23 \pm 0.12 \text{ nMol}$ to $2.51 \pm 0.05 \text{ nMol}$) of the total thiol groups in the patients after the hemodialysis. Figure 2 shows the results concerning to phenolic compounds, non-protein thiol groups and lipid peroxidation. The concentration of phenolic compounds decreased from $0.087 \pm 0.07 \text{ nMol}$ to $0.065 \pm 0.06 \text{ nMol}$ after the hemodialysis, whereas the concentration of non-protein thiol groups showed the tendency to decrease its concentration ($0.113 \pm 0.098 \text{ nM}$ to $0.095 \pm 0.028 \text{ nM}$). However, these alterations were not statistically significant ($p < 0.05$). The lipid peroxidation related to the reactivity of the lipid peroxides with thiobarbituric acid showed statistically significant increase in its values ($0.043 \pm 0.001 \text{ nMol}$ to $0.045 \pm 0.001 \text{ nMol}$) after the hemodialysis.

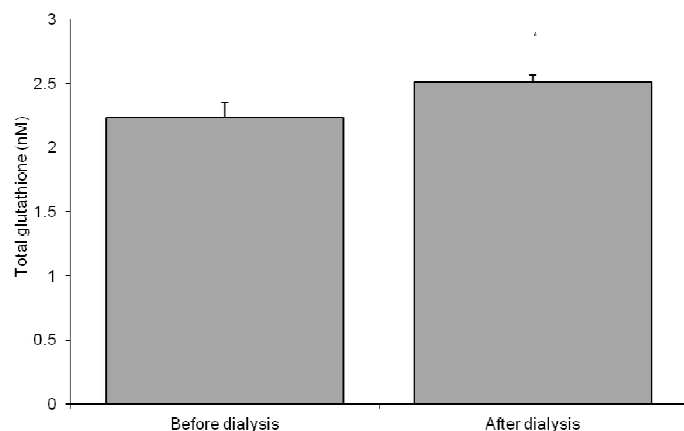


Fig. 1: Total glutathione in the patients undergo the hemodialysis.

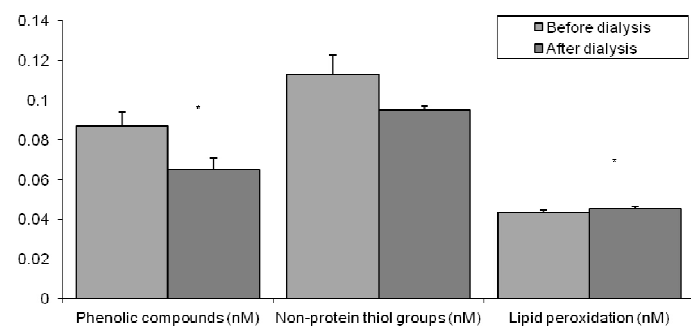


Fig. 2: Phenolic compounds, non-protein thiol groups and lipid peroxidation in the patients undergo the hemodialysis.

Figure 3 presents the results concerning the total, oxidized and reduced ascorbate, where was verified a slightly decrease in the concentration of total ascorbate ($0.082 \pm 0.011 \mu\text{mol}$ to

$0.075 \pm 0.007 \mu\text{mol}$) after the hemodialysis. However, the concentration of oxidized ascorbate showed a statistically significant increase ($0.052 \pm 0.0021 \mu\text{mol}$ to $0.064 \pm 0.0065 \mu\text{mol}$), combined with a statistically significant decrease in the concentration of reduced ascorbate ($0.03 \pm 0.012 \mu\text{mol}$ to $0.01 \pm 0.008 \mu\text{mol}$) after the hemodialysis.

Figure 4 presents the relation between oxidized/reduced ascorbate, where it is verified a statistically significant increase in the samples ($1.73 \pm 0.172 \mu\text{mol}$ to $6.4 \pm 0.812 \mu\text{mol}$) after the hemodialysis, indicating an increasing the uptake of the reduced form, increasing proportionally the oxidized form.

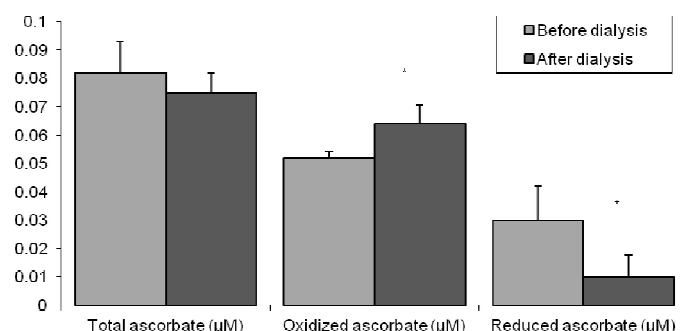


Fig. 3: Total, oxidized and reduced ascorbate in the patients undergo the hemodialysis.

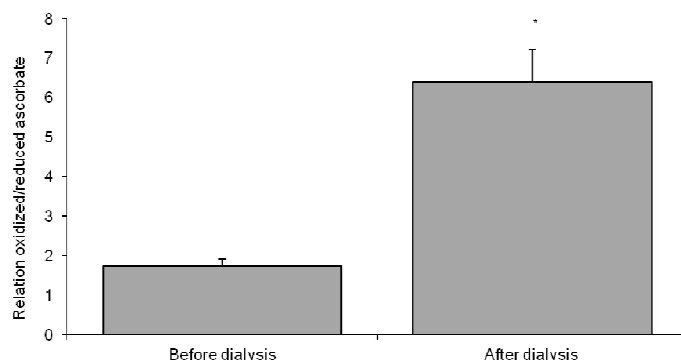


Fig. 4: Relation between oxidized/reduced ascorbate in the patients undergo the hemodialysis.

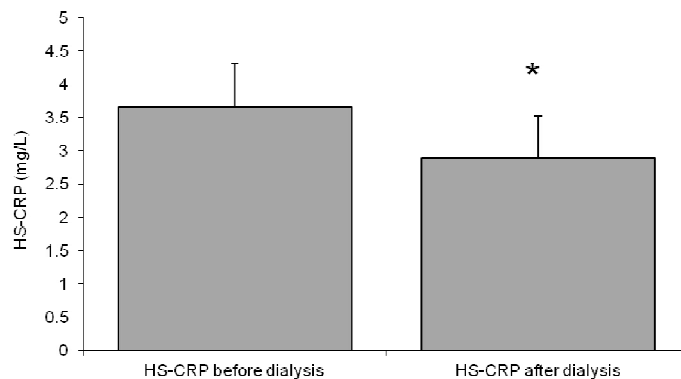


Fig. 5: High-sensitive C-reactive protein (HS-CRP) in the patients undergo the hemodialysis.

Figure 5 shows the results concerning the High-sensitive C-reactive protein (HS-CRP). From the results it is seen a

statistically significant increase in the concentrations of HS-CRP ($3.7 \pm 0.2 \text{ mg.L}^{-1}$ to $2.9 \pm 0.2 \text{ mg.L}^{-1}$) after the hemodialysis. Figure 6 present the results of nitric oxide (NO), where was verified the tendency to increase its value ($117.4 \pm 4.2 \text{ nmol}$ to $130.3 \pm 4.3 \text{ nmol}$), however without statistical significance ($p=0.292$). Figure 7 present the analysis of Pearson's correlation between difference in the concentration of HS-CRP and NO in the patients undergo the hemodialysis.

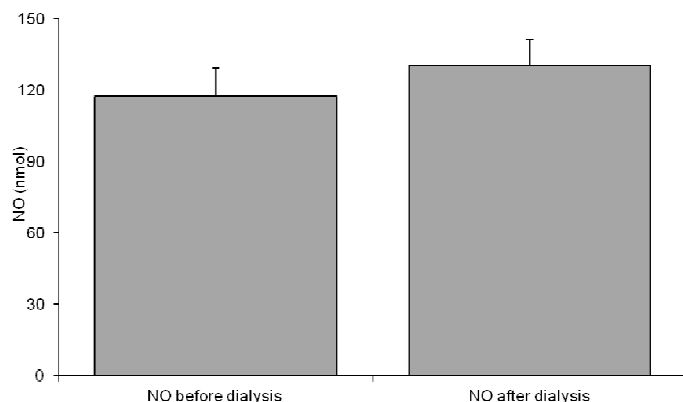


Fig. 6: Nitric oxide (NO) in the patients undergo the hemodialysis.

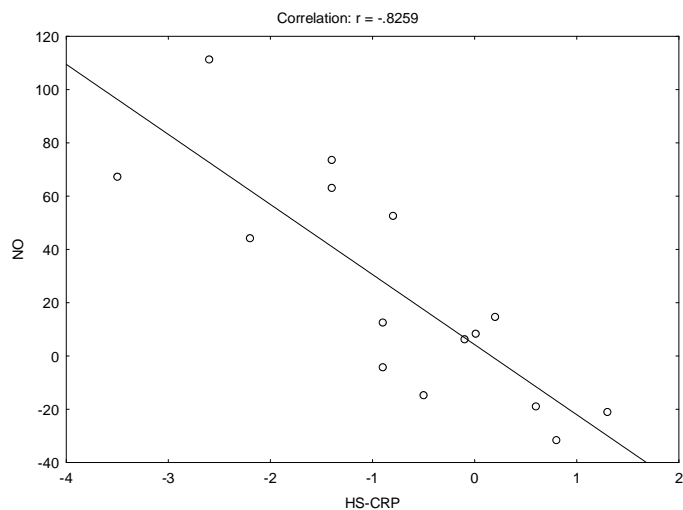


Fig. 7: Pearson's correlation between difference in the concentration of HS-CRP and NO in the patients undergo the hemodialysis.

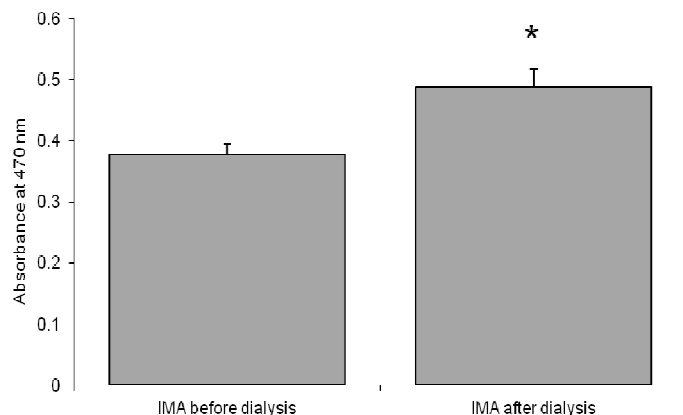


Figure 8. Ischemia-modified albumin (IMA) in the patients undergo the hemodialysis.

From the results, it is seen that the increase of NO concentration is statistically correlated with decrease of the HS-CRP at a significance level of 95%, presenting a correlation coefficient of -0.8259. This indicates a possible protector effect of the NO against endothelial damage induced by the hemodialysis.

Figure 8 presents the results obtained concerning the ischemia-modified albumin (IMA), where was verified a statistically significant increase in its value ($0.378 \pm 0.01 \text{ nm}$ to $0.488 \pm 0.02 \text{ nm}$) after the hemodialysis. This result indicates that the extracorporeal circulation increase the tissue ischemia, probably of the renal tissue induced by hemodialysis.

DISCUSSION

Some studies are reporting the increase of oxidant levels and reduction of the antioxidant in patients with chronic renal insufficiency undergoing hemodialysis (Dursun *et al.*, 2005). In addition, several symptoms of acute renal insufficiency can be due to overproduction of free radicals generated during ischemic reperfusion (Mafra *et al.*, 1999). According to Costa-Hong *et al.*, (2009) the oxidative stress and the endothelial dysfunction are promoters of atherosclerosis that presents high prevalence in the chronic renal disease.

From the results obtained in this study was verified a statistically significant increase in the total thiol groups associated with a reduction of non-protein thiol groups (indirect measurement of glutathione) after the hemodialysis. This result shows that during the hemodialysis occurs the consumption of glutathione that is a powerful antioxidant agent, predisposing the patient to a redox imbalance. Clermont *et al.*, (2000) also verified the marked depletion of antioxidant and a reduction of levels of thiol groups in patients after the hemodialysis, corroborating with the results obtained in this study.

The lipid peroxidation showed statistically significant increase in its values after the hemodialysis. The lipid peroxidation alter the fluidity and permeability of membranes (Belló-Klein *et al.*, 2004), promoting the releasing of hydrolytic enzymes as well as the production of toxic metabolites, leading to the cellular dead (Ferreira *et al.*, 1997). In addition, the results pointed out to the reduction of polyphenols, although there are no studies reporting the reduction of these compounds in patients undergoing hemodialysis. The consumption of antioxidants (including the polyphenols) can lead to complications arising from a clinical situation of oxidative stress, verified by the increased of the lipid peroxidation. The results related to the total, oxidized and reduced ascorbate pointed out to a statistically significant increase in the concentration of oxidized, combined with a statistically significant decrease in the concentration of reduced ascorbate after the hemodialysis. Based on these results, it is seen that the hemodialysis decreases the antioxidant protection by means of reduced ascorbate, with consequent increase in the content of oxidized ascorbate. The results of this work corroborating with those reported by Clermont *et al.*, (2000).

From the results concerning the endothelial adaptation it was seen that decrease the concentration of HS-CRP as inflammatory parameter showed to be inversely proportional to increase the concentration of NO. This result suggests the hemodynamic adaptation of patient during the hemodialysis. Some studies reported that leukocyte adhesion and the interaction leukocyte-endothelium, as well as, the releasing of cytotoxic compounds by leukocytes lead to a manifestation of reperfusion injury (Nunes *et al.*, 2006).

The NO did not showed alterations statistically significant in this study. However, it was verified the tendency to increase its concentration after the hemodialysis. The results obtained here corroborate with those of Marzi *et al.*, (1992), where stated that the concentration of NO should increase to restore the oxygen supply to ischemic tissue, since NO is an important vessel dilator. In this sense, the observed increase in the NO concentration due to caused a redox imbalance with consequent oxidative stress arising from ischemic reperfusion, decreasing the production of HS-CRP.

Several studies reported a close relation between the HS-CRP and the increase of cardiovascular risk (Ridker, 2007; Ridker *et al.*, 2007; Pearson *et al.*, 2003). HS-CRP is an inflammatory marker able to relieve the complement system, closely related to initial stages of formation of plaque atherosclerosis and also related to the stimulus of the synthesis of tissue factor by monocytes. The analysis of results concerning HS-CRP showed a significant reduction of its concentration, which can be associated with the increase of NO, due to endothelial adaptation of the patient undergoing hemodialysis.

Concerning the ischemia-modified albumin was verified a statistically significant increase in its value after the hemodialysis. This result indicates that the extracorporeal circulation increase the tissue ischemia, probably of the renal tissue induced by hemodialysis. Chicota *et al.*, (2008) reported the increase of IMA in patients with chronic renal disease, which was attributed to oxidative stress observed in these patients and also to the reduced levels of albumin, resulting in the increase of the concentration of free cobalt. According Roy *et al.*, (2006) the generation of free radicals can modify the N-terminal region of albumin, increasing the levels of IMA. Valentini *et al.*, (2007) reported that the renal dysfunction is accompanied of oxidative stress that consists in the damage of biologic structures by reactive oxygen species due to excessive generation and deficiency in defense mechanisms.

From the analysis of Pearson's correlation between the concentration of IMA and NO was not verified correlation (data not shown). Although NO is considered an inductor of oxidative stress, it showed an evident endothelial protective role due to reduction of levels of HS-CRP, however, did not contributed to increase the levels of IMA in this study.

From the results obtained here it was clear the biochemical effects caused by hemodialysis. To prevent or mitigate them, the patient should follow a few recommendations, balanced diet, nutritional counseling, intake of antioxidants and

citrus fruits, as well as, prevent the unnecessary use of oxidant medications (ex: acetaminophen).

CONCLUSIONS

Based on depletion of glutathione, polyphenols and reduced ascorbate, combined with the increase of oxidized ascorbate and lipid peroxidation can be concluded that the patients evaluated in this study presented a clinical situation of oxidative stress by oxygen metabolism (Reactive Oxygen Species). This conclusion was confirmed by the increase of levels of IMA in the plasma. In addition, NO showed an evident endothelial protective role due to reduction of levels of HS-CRP, however, did not contributed to increase the levels of IMA. It is known that these changes in the long-term can lead to serious consequences in the population as cardiovascular disease, aging and cancer. A new study focusing on the prevention of oxidative stress using different experimental models with antioxidants is valuable to improve the life quality of these patients.

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How to cite this article:

Valéria Dal Prá, Lais Biazus Bisol, Marisa L. Romani Paraboni, Luciano de Oliveira Siqueira. Evaluation of endothelial adaptation and parameters of oxidative stress in patients after hemodialysis sessions. *J App Pharm Sci*, 2013; 3 (10): 039-044.