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Effect of hemodialysis therapy on oxidative stress in patients with chronic renal failure

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SUMMARY

Background: *Cardiovascular disease remains the single most common cause of excess morbidity and mortality in end-stage renal disease (ESRD) patients and the traditional risk factors can't explain the high incidence of these events.*

New «non-traditional» risk factors are analysed in uremic patients and the increased oxidative stress is postulated to be an important contributor to uremic cardiovascular risk.

Methods: *In order to evaluate the effects of the hemodialysis treatment, a complete oxidative stress study was performed in fifteen uremic patients. Representative antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), together with oxidized/reduced glutathione ratio (GSSG/GSH) and other oxidation indicators including malondialdehyde (MDA) and 8-oxo-2´-deoxyguanosine (8-oxo-dG), were analysed to assess oxidative stress status in normal control volunteers and in uremic patients treated with hemodialysis (HD). In the latter group blood samples were taken prior and after HD to evaluate the effect of the session of HD over the oxidative markers.*

Results: *Low levels of antioxidant enzyme activities were observed in the uremic patients as compared with normal control subjects. HD treatment results in a significant recovery of these enzyme activities but remain lower as compared with control values. Levels of GSSG and GSH concentrations were increased and reduced respectively in uremic patients. These differences were even higher before the HD and were reduced upon treatment to levels closer to those observed in controls. MDA levels and 8-oxo-dG levels were also increased in uremic patients with the highest values observed in the pre-treated HD group. Eventhough HD treatment decreases the levels of oxidation products in mononuclear cells of uremic patients the values of the control group are not reached.*

Conclusions: *Our results suggest that hemodialysis by itself could correct the oxidative status in these patients. The possible mechanisms involved in the oxidative stress changes with the hemodialysis treatment will be discussed below.*

Key words: ***Oxidative stress. Hemodialysis. Antioxidant enzymes. Lipid peroxidation. GSH, 8-oxo-2´-deoxyguanosine.***

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EFECTO DEL TRATAMIENTO CON HEMODIÁLISIS SOBRE EL ESTRÉS OXIDATIVO EN PACIENTES CON INSUFICIENCIA RENAL CRÓNICA

RESUMEN

La enfermedad cardiovascular sigue siendo la principal causa de morbi-mortalidad de los pacientes con enfermedad renal crónica en tratamiento dialítico. Los llamados factores de riesgo tradicionales no son capaces de explicar la alta incidencia de estos sucesos por lo que cada vez más se está buscando nuevos factores de riesgo. Entre ellos el estrés oxidativo aumentado en estos pacientes podría ser un contribuyente importante en el riesgo cardiovascular.

Métodos: *Para evaluar los efectos del tratamiento con hemodiálisis hemos realizado un estudio completo del estrés oxidativo en 15 pacientes urémicos. Hemos analizado enzimas antioxidantes representativas como la superoxidodismutasa, catalasa y glutation peroxidasa, junto con el cociente entre glutation oxidado y reducido y otros indicadores de oxidación como el malonildialdehído y la 8-oxo-2´-deoxiguanosina. El análisis se ha realizado en pacientes hemodializados antes y después del tratamiento dialítico y se ha comparado con un grupo control de 16 voluntarios sanos.*

Resultados: *Encontramos un aumento de todos los parámetros de oxidación respecto a los del grupo control antes de la hemodiálisis con un descenso significativo tras la misma. Los parámetros antioxidantes son significativamente menores respecto a los de los controles con mejoría tras la hemodiálisis.*

Conclusiones: *Nuestros resultados sugieren que la hemodiálisis por sí misma podría corregir el estado pro-oxidante de nuestros pacientes. En el trabajo se analizan los posibles mecanismos implicados en los cambios en el estrés oxidativo con la hemodiálisis.*

Palabras clave: ***Estrés oxidativo. Hemodiálisis. Enzimas antioxidantes. Peroxidación lipídica. GSH, 8-oxo-2´-deoxiguanosina.***

INTRODUCTION

Patients with chronic renal failure (CRF) have three to five times higher likelihood of having a cardiovascular event than the general population,[1,2] and this likelihood is 3.5 to 50 times in hemodialysis patients.[3]

Currently the importance in these patients of classical risk factors such as anemia, hypertension, diabetes, etc., and the association of atherosclerotic and inflammatory phenomena linked to an increase in oxidative stress (OS) is highlighted.[4,5]

Oxidative stress arises from a misbalance between the production of oxidation-derived products and the organism defense mechanisms to clear them. In normal conditions, both systems are in a steady state in such a way that an increase in oxidative products is followed by a greater production by anti-oxidant systems. This balance always favors the anti-oxidant arm so that there is a safety zone.

There is increasing evidence about the presence of oxidative stress in chronic renal failure patients, and particularly in those submitted to hemodialysis therapy. This seems to be due to multiple factors including an increase in the production of agents from oxidative metabolism (oxygen-derived substances generated by activated leukocytes, transition metal compounds, and other toxins of different molecular weight), and a decrease in anti-oxidant defenses. Besides, the use of low biocompatible membranes and purity of dialysis water[6,7] has an influence on oxidative stress. OS generated in a physiologic or pathologic way harms cellular constituents including membrane lipids, proteins, and DNA. As for the study of nuclear damage, it has been observed that 8-hydroxy-2´-deoxiguanosine (8-OH-dG) is the most DNA abundant oxidative product, reflecting small oxidative changes in nuclear damage and, therefore, it is considered a new marker for oxidative damage research.[8]

In the present study we aim at analyzing, in a population of CRF patients on hemodialysis, both antioxidant defense markers in leukocytes and membrane lipid peroxidation thought the determination of malonildialdehyde (MDA) and nuclear damage by 8-OH-dG changes, and to observe the effect that an HD session has on these markers.

PATIENTS AND METHODS

Patients

Patients included in the study come from the hemodialysis unit of our Hospital. Those meeting inclusion criteria were invited to participate and informed written consent was required. In order to avoid confounding factors, we excluded all patients that were diabetic, smokers, aged more than 70 years, that had received intravenous iron for the last 3 months, those treated with ACEIs, and those on dialysis therapy for less than 3 months. All patients were checked for C reactive protein and we excluded those suspected of having inflammatory or infectious conditions at the time of the study.

Finally, the group was comprised by 15 patients, 10 men and 5 women, with a mean age of 56.90 years (39-70) and a mean duration of therapy of 33.71 months.

The causes leading to CRF were the usual ones in an HD population, excluding diabetes: glomerulonephritis in 4 cases, tubulointerstitial nephritis in 3 cases, another 3 cases with vascular origin, 1 polycystic disease, 1 of unknown origin, and 3 from other causes.

All patients had a standard HD therapy with polysulphone or polyacrilonitrile hemofilters, for 12 hours/week, achieving adequate dialysis dosing parameters (minimal Goth's Kt/V of 1.2).

Sixteen apparently healthy subjects, with similar age and demographic characteristics were used as controls.

Methods

The following anti-oxidant markers were determined in lymphocytes of both patients and controls: catalase (CAT), reduced gluthathione (GSH), gluthathione peroxidase (GPX), superoxide dismutase (SOD), and the oxidative markers oxidized gluthathione (GSSG), malonildialdehyde (MDA), GSSG/GSH ratio, and 8-OH-dG, as an index of specific nuclear damage. Determinations were performed at the beginning and at the end of the HD session in the middle of the week.

One mL of blood is centrifuged at 13,000 rpm for 30 min. The supernatant is transferred to a new tube and stored at –20º C until MDA determination. An equivalent volume of distilled water is added to the cellular pellet and the hemolyzed sample is stored at 4º C for 2 hours. We used 200 mL aliquots for analyzing hemoglobin and gluthathione peroxidase (GPX). The remaining 300 mL are mixed to 3:5 chloroform/ethanol (vol:vol) and centrifuged at 13,000 rpm for 30 min. The supernatant is used to determine reduced gluthathione (GSH), with previous addition of 10 mL of 20% PCA solution , and catalase (CAT) and superoxide dismutase (SOD).

Whole heparanized blood is diluted in saline and mononuclear cells are isolated by centrifugation with Ficoll-Hypaque followed by three washings.[9] The percentage of lymphocytes in the cellular suspension is 80-90%. The cellular reduced gluthathione (GSH) content is determined by the previously described method.[10] For oxidized gluthathione (GSSG) analysis, the samples are treated and analyzed with high resolution liquid chromatography (HPLC), according to the previously described method.[11,12] MDA is also analyzed by HPLC.[13] The protein content in the sample is measured by Bradford's method.[14]

Total SOD activity was measured by McCord and Fridovich method,[15] based on the ability of superoxide anions to reduce oxidized cytochrome C and inhibition of this reaction in the presence of the tissue enzyme. Catalytic breakdown of xanthine by xanthine oxidase enzyme is used as a source of superoxide anions. The decrease in cytochrome C reduction is measured by means of 546 nm wavelength spectrophotometry. An SOD unit is defined as the concentration of enzyme able to decrease by 50% the rate of reduction of oxidized cytochrome C, the result being expressed in U/mg of protein.

Catalase activity (CAT) is determined by Clairbone's method[16] based on the breakdown of hydrogen peroxide in water and oxygen by CAT, measured by means of spectrophotometry. It is expressed in U/g of protein.

Gluthathione peroxidase (GPX) activity was determined by Gunzler and Flohe method.[17] The oxidized gluthathione (GSSG) yielded by GPX action is immediately and continuously reduced due to an overactive gluthathione reductasa, providing a constant level of GSH.

Cellular DNA was isolated by Gupta's method,[18] with Muñiz's modification,[19], in which chloroform isoamyl alcohol (24:1) is used instead of phenol for protein segregation. Isolated DNA is two times washed with 70% ethanol, it is then dried and diluted in 200 mL of 10 mmol/L of Tris/HCl, 0.1 mmol/L of EDTA, and 100 mmol/L of NaCl (pH 7.0) for enzymatic digestion.

Once the samples have been digested, they are filtered through 0.2 mm microfilters before injecting them into the HPLC system. These samples are separated through a chromatography column (Waters ODS). The amount of 8-oxo-dG and of dG in digested DNA is measured by means of an electrochemical detector and UV absorbance, respectively,

as previously described.[20] The concentration of this base is registered and calculated by a software, being expressed as picomoles of 8-oxo-dG/ 10^5 dG.

Statistical analysis

Statistical analysis was done by means of the statistical package SPSS 11.5. Values are presented as mean and standard deviation, and 95% confidence interval. Normality of the distribution was checked for each variable (one sample Kolmogorov- Smirnov test). The difference between the means from the different groups was checked by ANOVA with one post-hoc Bonferroni's fitness factor in case of homogeneity of variances, or by Dunnet's T3 in the opposite case. Chi-squared was used for qualitative variables, and Mann Whitney's for group differences, in spite of the normal distribution for most of the variables, due to the sample size. Bi-variate multiple correlations were performed between the different variables in the whole group of patients by Spearman's Rho.

RESULTS

Demographical characteristics of controls and patients are shown in table I. We did not observe differences between both groups by age, gender, weight, and determinations of pre-albumin, hemoglobin, and ferritin. All patients had C reactive protein lower than 5 mg/dL.

The analysis of anti-oxidant enzymatic activities in lymphocytes shows significant differences between controls and patients on hemodialysis (table II). The values before hemodialysis are significantly decreased as compared to controls. HD therapy produces a significant increase of the three measured enzymes, but the case of CAT it does not reach significant differences with healthy controls.

Intracellular concentration of GSH is significantly reduced in patients before HD. After this therapy, there is a recovery of this tripeptide, although still with values below those in the control group. GSH decrease is accompanied by an increase of its oxidation product GSSG, and therefore there is an increase of the GSSG/GSH ratio, particularly evident in patients before HD, indicating an oxidative status in these individuals during the non-dialysis periods (table III).

We also found an increase in lipid peroxidation in uremic patients, expressed as the increase pro-

Table I. General characteristics of the studied population

Variables	Controls (n = 16)	Patients on HD (n = 15)	p
Age (years)	56.18 (35-70)	56.90 (39-70)	NS
Gender (M/F)	11/5	10/5	NS
Pre-albumin (mg/dl)	38.09 ± 2.07	35 ± 5.52	NS
Ferritin (µg/L)	258.4 (16.9-709)	411.8 (239-650)	NS
CRP	1.20	1.10	NS
Comorbidity index (Charlson's index)	0.22	3.91	< 0.05

Ferritin values are expressed as medians with range; the remaining continuous variables are expressed as mean ± standard deviation.
CRP: C reactive protein.
We used Charlson's index as a comorbidity index.

Table II. Antioxidant enzymatic activities in lymphocytes in normal subjects and in uremic patients, and effect of dialytic therapy

	Controles (n = 16)	Patients on HD (n = 15)	
		bHD	aHD
CAT (U/g prot)	276.97 ± 33.29 (205.99-347.94)	110.36 ± 5.94[a] (97.75-122.97)	251.44 ± 18.67[b] (211.86-291.02)
GPx (U/g prot)	57.18 ± 2.04 (52.82-61.53)	25.93 ± 1.62[a] (22.50-29.37)	44.40 ± 1.77[a,b] (40.63-48.16)
SOD (U/mg prot)	5.72 ± 0.15 (5.0-6.05)	1.69 ± 0.18[a] (1.30-2.08)	3.75 ± 0.21[a,b] (3.29-4.20)

The values are expressed as mean ± SD (95% confidence intervals).
bHD: before hemodialysis.
aHD: after hemodialysis.
[a]p < 0.005 *versus* controls.
[b]p < 0.005 *versus* before hemodialysis.

Table III. Gluthathione levels and GSSG/GSH ratio in lymphocytes of normal subjects and uremic patients, and effect dialytic therapy

	Controls (n = 16)	Patients on HD (n = 15)	
		bHD	aHD
GSSG (nmoles/mg prot)	0.15 ± 0.02 (0.11-0.20)	0.59 ± 0.02 [a] (0.53-0.65)	0.38 ± 0.01[a,b] (0.35-0.42)
GSSG/GSH x 100	0.60 ± 0.07 (0.43-0.76)	4.26 ± 0.26 [a] (3.71-4.82)	1.86 ± 0.10[a,b] (1.64-2.08)
GSH (nmoles/mg prot)	26.19 ± 1.24 (23.54-28.84)	14.23 ± 0.48 [a] (13.21-15.24)	21.15 ± 0.55[a,b] (19.98-22.31)

The values are expressed as mean ± SD (95% confidence intervals).
bHD: Before hemodialysis.
aHD: after hemodialysis
[a]p < 0,005 *versus* controls.
[b]p < 0,005 *versus* before hemodialysis.

duction of MDA in circulating cells. HD therapy leads to a decrease in this peroxidation.

As for nuclear damage, we found increased levels of 8-oxo-dG in HD patients, which improves after HD remaining higher than values in healthy controls (table IV).

We studied the correlation between 8-oxo-dG and the remaining analyzed parameters, and we found a significant negative correlation with GSH, GPX, and SOD, and a positive correlation with MDA, GSSG, and the GSSG/GSH ratio (table V).

DISCUSSION

It is clearly shown that CRF patients have increased OS produced by decreased antioxidant defenses and an increase in pro-oxidant factors.[4,21,22,23] Several pathophysiologic explanations have been claimed; some attribute it to malnutrition and hypoalbuminemia having in theses cases low availability of «thiol»; others to «uremic status» itself with solute retention that may favor their pathogenicity; and others to the association of comorbid factors such as advanced age, diabetes, and inflammatory and infectious phenomena.[24,25] Besides, when these uremic patients receive treatment with an extrarenal depurative technique, as is HD, OS is promoted by several reasons among which the usage of low-biocompatible synthetic membranes and the lack of ultrapure dialysis water stand out.

To assess the existence of OS, the ideal would be to measure free radicals produced (O_2^-, OH^-, H_2O_2, etc.), but this is technically very difficult due to the very short half life of these products and it would

Table IV. Lipid peroxidation and DNA damage in normal subjects and uremic patients, and effect of dialytic therapy

	Controls (n = 16)	Patients on HD (n = 15)	
		bHD	aHD
MDA (nmoles/mg prot)	0.15 ± 0.018 (0.11-0.19)	0.50 ± 0.03 [a] (0.42-0.58)	0.34 ± 0.01 [a,b] (0.31-0.37)
8-oxo-dG/10^6 dG	2.97 ± 0.16 (2.62-3.32)	6.62 ± 0.23 [a] (6.13-7.11)	5.10 ± 0.21 [a,b] (4.63-5.56)

The values are expressed as mean ± SD (95% confidence intervals).
bHD: before hemodialysis.
aHD: after hemodialysis.
[a]p< 0,005 *versus* controls.
[b]p < 0,005 *versus* before hemodialysis.

Table V. Correlation between 8-OH-dG and other oxidative markers in dialysis patients

	r*	p
GSH	- 0.552	0.001
GSSG	0.330	0.035
GSSG/GSH x 100	0.552	0.001
SOD	- 0.531	0.001

* Spearman's correlation coefficient.

be rather unspecific.[26] Thus, the best is to measure the results of the damage inflicted by free radicals.

When free radicals overwhelm the antioxidant barrier, they become available for interacting with phospholipidic structures producing *lipid peroxidation*. One of the major components of this reaction is the production of malonildialdehyde (MDA), which identification would allow us having an idea of the magnitude of lipid peroxidation. The results found in our patients are in agreement with those of many other authors regarding increased lipid peroxidation.[27,28] However, it is important to point out that not all authors determine MDA as the marker of lipid peroxidation, but other products such as TBARS (thiobarbituric acid reactive substances)[29] or even myeloperoxidase.[30] What seems to be the only available technique sensitive enough, so far, has recently come into place; it consists of post-column chemiluminescent detection after segregation by high performance liquid chromatography (HPLC).[31]

The action of free radicals (FR) on proteins produces proteinic carboxyls that are not determined in our laboratory. Finally, FR may act on nuclear DNA producing structural changes so that a guanine base is oxidized to guanosine yielding a the *8-oxo-deoxiguanosine* compound that may quantified through HPLC. The emergence of this oxidized base has a crucial relevance since it reflects the intensity and specificity of oxidative damage, besides of having a mutagenic potential.[32]

In our patients, before having HD, which would represent their «baseline status», we found significantly increased 8-oxo-dG levels as compared to controls. Besides, we found a significant correlation with most of the other biomarkers. From these results it may be inferred that DNA damage runs parallel to the damage to cellular metabolism, which would be in agreement with the conclusion established by Tarng *et al.*[33] in which he states that 8-oxo-dG increase in lymphocytes is an integrated effect of the deficient cellular redox status due to a decrease in antioxidant defenses and an increase in free radical production.

Gluthathione is a tripeptidic thiol found in the inside of all animal cells and likely is the most important cellular antioxidant. Oxidized gluthathione (GSSG) is highly toxic to cells so that the organism tends to reduce GSSG to GSH through gluthathione reductasa. Thus, determining GSSG/GSH ratio is considered a reliable estimate of the degree of cellular oxidative stress.[31,34]

As for antioxidant mechanisms, we have determined the enzymatic triad compounded by *superoxide dismutase* (SOD), *catalase* (CAT), and *gluthathione peroxidase* (GPX). Our patients had deceased levels of the three enzymes before having HD.

The effect that an HD session may have on OS is a controversial issue. For some authors, HD would aggravate OS due to activation of inflammatory cells caused by the use of bioincompatible membranes and net losses of soluble antioxidants in water[8,35,36] or by generation of free radicals.[37,38] On the contrary, decreased MDA with HD has also been pointed out, and the work by Himmelfarb *et al.*[41,42] stands out, showing the beneficial effects of HD on major plasma amino-thiols (cysteine, homocysteine, cysteinyl glycine, and gluthathione), which are important markers of oxidation. Biasioli *et al.*[43] studied the effect produced by several types of membranes used during hemodialysis and they observe a decrease in OS throughout HD with more biocompatible membranes; they also performed post-dialysis determinations showing an improvement of the different markers, and 30 minutes after completing HD with MDA values becoming similar to those pre-dialysis.

Our results obtained from HD patients after one dialysis session are in agreement with these latter findings, and show that values of both antioxidant and pro-oxidant markers improve after HD, although still being significantly different from those in controls. In this regard, Roselaar *et al.*[44] showed the presence of a dialyzable oxidant in the plasma of patients on HD and, thus, they found an improvement of OS after HD.

It is necessary to perform prospective studies in HD-treated patients with pre-, post-dialysis determinations and 24 hours thereafter in order to perform an oxidative profile in-between two hemodialysis sessions.

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