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Analysis of oxidative stress in patients on on-line hemodiafiltration

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SUMMARY

Patients with chronic renal disease have a very high mortality due to cardiovascular disease. However, the traditional risk factors are not the only one explanation. Nowadays, there are new risk factors becoming, and one of these is the oxidative stress. Besides today we know that when these patients receive haemodialysis are being exposed to an additional oxidative stress. The aim of this study was to mesure and to compare the degree of oxidative stress in two groups of patients on different dialysis techniques: a) On-Line Haemodiafiltration three times / week (OL-HDF). b) Daily Om-Line haemodiafiltration (six times/week) (dOL-HDF) We studied 9 patients with chronic renal disease stage 5 on hemodialysis. They all were men, with a medium age of 72,5 ± 6 years. Five patiens were on dOL-HDFand four on tOL-HDF. Glutathione (GSH) concentration of patients on dOL-HDF before dialysis was 742 ± 153 nmol/ml and post-dialysis de 878 ± 223. Blood GSSG concentration before and after dialysis was 34 ± 14 nmol/ml y 137 ± 74 nmol/ml (p < 0,03). GSSG/GSH ratio pre-dialysis was 58 ± 10 and post-dialysis 169 ± 65 (p < 0,03). In OL-HDF group GSSG concentration and the ratio GSSG/GSH also increased in a significative way from 99 ± 45 nmol/ml to 179 ± 66 nmol/ml, and from 161 ± 99 to 337 ± 143 (p < 0,05). We also found differences in pCR concentrations between both groups; 3 ± 1,4 g/l in dOL-HDF and 8,75 ± 5,8 g/l in HDF OL (p < 0,05). We did not find differences between xatine-oxidase activity before and after hemodialysis and between groups. ***In conclusion,*** *patient with terminal chronic renal disease on OL-HDF receive an additional load of oxidative stress, as the increase in GSSG/GSH ratio in both groups shows. However patients on dHDF-OL shows low ratios GSSG/GSH post-hemodialysis and low pCR concentrations, and maybe this could be explained because daily on line haemodiafiltration improves purification of inflammatory mediators. Clue words: Hemodialysis, oxidative stress, glutathione, gssg/gsh ratio, xantine oxidasa.*

Key words: ***On-line hemodiafiltration. Oxidative stress. Glutathione. GSSG/GSH ratio. Xantine oxidasa.***

ANÁLISIS DEL ESTRÉS OXIDATIVO EN PACIENTES EN HEMODIAFILTRACIÓN EN LÍNEA

RESUMEN

Los pacientes afectos de enfermedad renal crónica presentan una elevada morbimortalidad debido a enfermedades cardiovasculares. Sin embargo, la elevada pre-

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*sencia de estas enfermedades no puede explicarse únicamente por los factores de riesgo tradicionales. En la actualidad, se considera la existencia de factores de riesgo emergentes, entre los que se encuentra el estrés oxidativo. Además se sabe que cuando reciben tratamiento con hemodiálisis, se ven sometidos a un estrés oxidativo adicional. El objetivo de este trabajo ha sido analizar y comparar el grado de estrés oxidativo en dos grupos de pacientes urémicos dializados con diferentes técnicas dialíticas: a. Hemodiafiltración on-line 3 veces/semana (HDFOL). b. Hemodiafiltración on-line diaria 6 veces semana(HDFOLD). Se estudiaron 9 pacientes afectos de enfermedad renal crónica terminal (Estadio 5), todos ellos varones con una edad media de 72,5 ± 6 años. Cinco pacientes pertenecían al grupo de HDFOLD y cuatro al grupo de HDFOL tres veces por semana. Los pacientes del grupo de HDF-OLD presentaban las siguientes concentraciones de glutatión reducido (GSH) en sangre, pre-diálisis de 742 ± 153 nmol/ml y post-diálisis de 878 ± 223, sin detectarse diferencias significativas entre ambos. Las concentraciones pre y post-diálisis de glutatión oxidado (GSSG) en sangre eran de 34 ± 14 nmol/ml y 137 ± 74 nmol/ml respectivamente (p < 0,03). Los cocientes GSSG/GSH obtenidos fueron: pre-diálisis de 58 ± 10 y post-diálisis 169 ± 65, con diferencias entre ambos valores (p < 0,03). Los pacientes del grupo HDF-OL 3 veces/semana también presentaron un incremento significativo de la concentración de GSSG y del ratio GSSG/GSH tras la sesión de diálisis, de 99 ± 45 nmol/ml a 179 ± 66 nmol/ml y de 161 ± 99 y a 337 ± 143, respectivamente (p < 0,05). La mediana de los valores de proteína C reactiva eran de 4,12 g/l en el grupo HDFOLD y 7,7 g/l en grupo de HDFOL (p < 0,05). No encontramos diferencias estadisticas en la actividad de la xantina oxidasa entre grupos ni tras la sesión de hemodiálisis. **En resumen,** podemos concluir que los pacientes afectos de enfermedad renal crónica terminal que reciben tratamiento sustitutivo se encuentran sometidos a un estrés oxidativo adicional, como muestra el incremento en los ratios GSSG/GSH en ambos grupos. Sin embargo los pacientes en el grupo HDFOLD presentan cocientes GSSG/GSH post-hemodiálisis y valores de PCR inferiores, lo que sugiere que la hemodiálisis diaria podría mejorar la depuración de medidores inflamatorios.*

Palabras clave: ***Hemodiafiltración on-line. Estrés oxidativo. Glutatión. Cociente GSSG/GSH. Xantine oxidasa.***

INTRODUCTION

Chronic renal failure is associated with classical cardiovascular risk factors, as well as with factors derived from the disease itself, such as chronic volume expansion, impairments of the calcium-phosphate metabolism, hyperhomocysteinemia, and permanent oxidative stress-associated inflammatory state. These non-classical factors are becoming very important since they may be in part responsible of the high incidence of cardiovascular events in such patients.

Patients with chronic renal disease are characterized by the presence of balance impairment between pro-oxidative substances and anti-oxidant factors.[1]

Cells are protected against oxidation by a complex series of anti-oxidants. Oxidative stress occurs when there is an unbalance between pro-oxidant and anti-oxidant substances favoring the former.[2]

The main anti-oxidant substances in biological systems are oxygen reactive species (ROS), among which superoxide anion ($O2^-$), hydrogen peroxide (H2O2), hydroxyl radical (OH^-), oxygen singlet, and hypochlorous acid stand out.[3]

Oxidative stress is considered an important etiological factor in many human pathological processes. It has been related with carcinogenesis, arteriosclerosis, arterial hypertension, neurodegenerative diseases, renal failure, acquired immunodeficiency syndrome, and long-term diabetes complications.

There are currently accurate parameters and appropriate techniques to quantify oxidative stress. Among the most frequently used ones there are:[5] Reduced gluthathione (GSH), oxidized gluthathione (GSSG), malondialdehyde (MDA) levels, 8-OH-deoxi-guanosin, and xanthine oxidase (XO).[4-6]

It is known that patients presenting end-stage chronic renal disease are submitted to additional oxidati-

Table I. Pre and post-dialysis GSH, GSSG, GSSG/GSH and xanthine oxidase activity in DOLHDF and OLHDF three times per week

	GSH (nmol/mL)	GSSG (nmol/mL)	GSSG/GSH	Xanthine oxidase (mU/mL)
DOLHDF				
Predialysis	741.5 ± 153.47	34 ± 14	58 ± 10	0.36 ± 0.1
Post-dialysis	878 ± 227	138 ± 74*	169 ± 65**	0.33 ± 0.7
OLHDF				
Predialysis	723 ± 344	99 ± 45	161 ± 99	0.52 ± 0.24
Post-dialysis	564 ± 233	179 ± 66*	337 ± 143**	0.34 ± 0.05

GSH: Reduced gluthatione. GSSG: Oxidized gluthatione. DOLHDF: Daily on-line hemodiafiltration. OLHDF: Hemodiafiltration on-line 3 times weekly. *p < 0.05 *vs* pre-dialysis. **p < 0.05 *vs* pre-dialysis.

ve stress when treated with hemodialysis. In this sense, there are more and more data implicating ROS in the development of long-term hemodialysis complications such as amyloidosis, atherosclerosis, and cardiovascular diseases in general.[7]

Age, time on dialysis, diabetes mellitus, inflammation, hemo-incompatibility of dialysis membranes, diffuse loss of antioxidant substances such as vitamins (ascorbic acid, tocopherols), the use of supplementary therapies such as intravenous iron, have been postulated as possible inducers of oxidative stress in hemodialysis patients.[8-10]

Several strategies have been proposed to minimize oxidative stress during dialysis and trying to prevent its effects. For instance, the use of biocompatible membranes and ultrapure dialysis fluids, the use of membranes that might achieve clearing or adsorbing inflammatory mediators from plasma, hemolipodialysis, a new technique using vitamin C-containing dialysis fluids, vitamin E-containing liposomes, as well as changing the frequency of weekly dialysis sessions.[11-14]

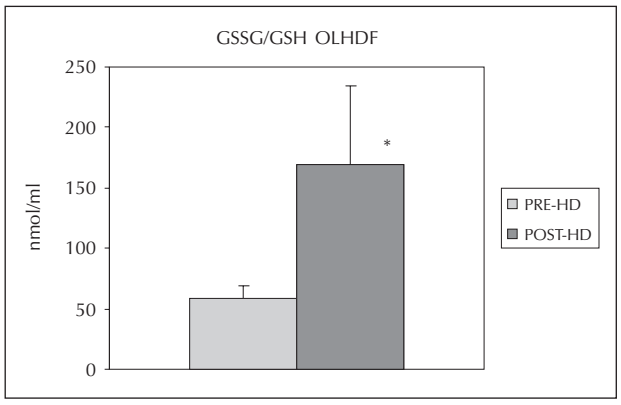

*Fig. 1.—Pre and post-hemodialysis GSSG/GSH ratio in the group on daily hemodialysis. *P < 0.05* vs *pre-HD.*

OBJECTIVE

The aim of this work was to analyze and compare the degree of oxidative stress in tow groups of uremic patients dialyzed with on-line hemodiafiltration, with different frequency:

a) On-line hemodiafiltration 3 times weekly (OLHDF).
b) Daily on-line hemodiafiltration 6 times weekly (DOLHDF).

MATERIAL AND METHODS

Nine patients meeting the following criteria were included into the study:

- Being on replacement therapy for at least 6 months.
- Age 35-85 years.
- Male gender, so as to prevent inter-sex variability.
- Ferritin levels < 600 pg/mL and Hb > 10 g/dL.
- Residual urine output < 300 mL/min.

Exclusion criteria were as follows: severe hyperparathyroidism (PTH > 500), active systemic inflammatory or infectious disease, not controlled neoplasm, immunosuppressive therapy.

The mean age of the patients was 72.5 ± 6 years.

Five patients were in the daily OLHDF group, and four patients in the OLHDF three times weekly group.

Patients in the DOLHDF received dialysis 6 times/week, 143 ± 9 minutes per session on average, with a convection volume with subsequent post-dilution re-infusion of 15.4 liters/session. The time on dialysis program was 14 ± 0.8 years.

Patients on OLHDF three times per week received dialysis 262 ± 15 min per session on average, with a

Table II. Main laboratory parameters

	Hb	Urea	Creat.	Alb	PTH	Ferritin	TSI%
DOLHDF	13 ± 2	69.5 ± 1	6 ± 1	3.7 ± 0.2	269 ± 144	507 ± 572	36 ± 18
OLHDF	13.7 ± 1	92 ± 32	8 ± 2	3.7 ± 0.2	147 ± 119	572 ± 580	38 ± 14

DOLHDF: Daily on-line hemodiafiltration. OLHDF: «on-line» hemodiafiltration 3 times weekly. Hb: Hemoglobin g/dL. Creat: Creatinine mg/dL. Alb: Albumin g/dL. PTH: Parathormone pg/mL. TSI: Transferrin saturation index %.

convection volume with subsequent post-dilution reinfusion of 22 liters/session, and time on hemodialysis of 9 ± 5 years.

The etiologies of chronic renal disease for the different patients was: vascular nephropathy 5 cases, polycystic renal disease 2 cases, and diabetic nephropathy and of unknown origin, one case each.

The composition of the dialysis fluid was: Sodium 140 mEq/L, potassium 3 mEq/L, calcium 3 mEq/L, magnesium 1.5 mEq/L, chlorine 117 mEq/L, bicarbonate 30 mEq/L, acetic acid 2 mEq/L. The dialyzers used were 1.89 m^2, high-permeability polysulfone in the five patients on DOLHDF, of 2 m^2 in 3 patients with OLHDF three times weekly, and 1.89 m^2 in the remaining patient.

All patients received a sucrose-iron perfusion during dialysis, and at the end of the session they received intravenous erythropoietin. Besides, all patients chronically received post-dialysis vitamin supplements three times per week, including vitamins B1, B6, B12, H, and 200 mg of vitamin C and folic acid.

*Blood samples:*_Twenty to twenty-five milliliters of blood from the arterial circuit of the patient were drawn and put in EDTA-anti-coagulated tubes (purple cap) before and immediately after dialysis session. The samples were immediately deproteinized for determination of oxidized and reduced gluthathione (*see* methods). After deproteinization, the samples were ultracentrifuged at 15,000 g for 15 minutes, and then the supernatants were stored at -40º C.

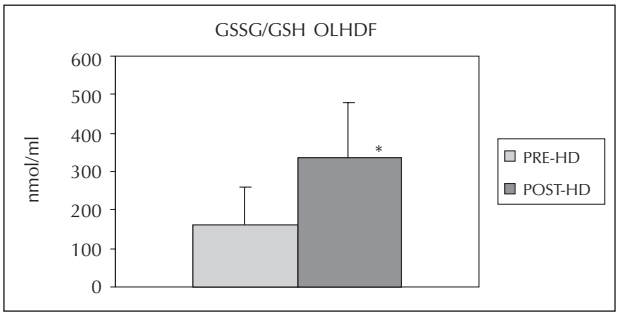

*Fig. 2.—Pre and post-hemodialysis GSSG/GSH ratio in the group on OLHDF 3 times weekly. *p < 0.05 vs pre-HD.*

METHODS

Plasma levels of oxidized gluthathione, reduced gluthathione, and the activity of xanthine oxidase were determined. We used the modified Brigelius gluthathione-S-transferase method to determined reduced gluthathione.[15] We used the method by Asensi et al. to determined oxidized gluthathione (GSSG).[16] We used the Kit (A-22182) Amplex® red Xanthine/ Xanthine oxidase to determine the activity of xanthine oxidase.

In order to compare the dialysis dose, we used the Casino «eKR» formula, which is equivalent to renal urea clearance, allowing for quantification of total urea clearance (renal and dialytic) according to time for any kind of dialysis and independently of its duration.[17]

The patients were informed and signed a written consent document to participate in the study.

Statistical analysis

The results are expressed as the arithmetic mean ± standard deviation. The analysis of variance (ANOVA), in the first place, and the Student's t test, have been used to compare quantitative parameters. The correlation between numerical variables was determined by the Pearson's method. A p value < 0.05 was considered statistically significant.

RESULTS

The patients from the DOLHDF group had a blood level of reduced gluthathione of 742 ± 153 and 878 ± 223 nmol/mL, pre- and postdialysis, respectively (NS).

The pre and post-dialysis blood levels of oxidized gluthathione were 34 ± 14 nmol/mL and 137 ± 74 nmol/mL, respectively, the difference between both

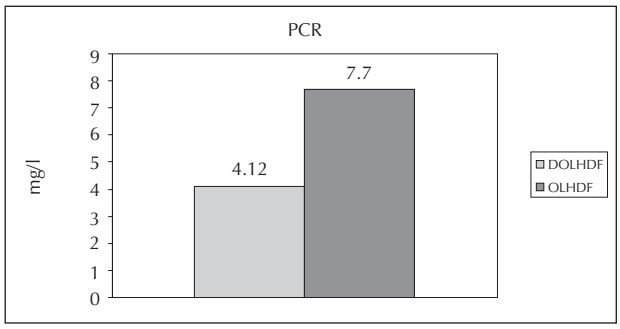

Fig. 3.—Median C reactive protein level in the groups of OLHDF 3 times weekly and daily OLHDF.

being statistically significant ($p < 0.03$). The GSSG/GSH ratios obtained were 58 ± 10 nmol/mL and 169 ± 65 nmol/mL, pre and post-dialysis, respectively ($p < 0.03$) (fig. 1).

On the other hand, patients from the OLHDF 3 times weekly presented a pre-dialysis GSH level of 723 ± 344 nmol/mL and after the session of 564 ± 233 nmol/mL (NS).

About oxidized gluthathione, the pre and post-dialysis levels were 99 ± 45 nmol/mL and 179 ± 66 nmol/mL, respectively ($p < 0.05$). The pre-dialysis and post-dialysis GGSG/GSH ratios obtained were 161 ± 99 and 337 ± 143, respectively ($p < 0.05$) (fig. 2).

We did not find any significant difference between the groups for pre and post-dialysis GSSG levels.

There was a trend in the DOLHDF group to have lower post-dialysis GGSG/GSH ratios ($p = 0.05$).

About the activity of xanthine oxidase, pre-dialysis values were 0.36 ± 0.1 mU/mL and 0.52 ± 0.24 mU/mL in the DOLHDF and OLHDF three times weekly groups, respectively. The post-dialysis activity of the enzyme was 0.33 ± 0.8 mU/mL and 0.34 ± 0.05 mU/mL, respectively (table I).

We did not observe significant differences between the groups in any of the following parameters: hemoglobin (Hb), urea, creatinine, albumin (alb), total proteins (TP), PTH, ferritin, transferrin saturation index (TSI), or uric acid (table II).

Patients in the DOLHDF group presented an eKR of 28.1 ± 2.3 mL/min and those in the OLHDF 3 times weekly group of 18.7 ± 4.3 mL/min ($p < 0.05$).

The median value for C reactive protein was 4.12 g/L in the DOLHDF group and 7.7g/L in the OLHDF 3 times weekly group ($p < 0.05$) (fig. 3).

The administered doses of iron and EPO were 10 mg/session and 2000 IU/week in the DOLHDF group, and 12.5 mg/session and 5500 IU/week in the OLHDF group. There were no significant differences between the groups for iron therapy, but yes with regards to the erythropoietin dose administered ($p < 0.05$).

The heparin dose (sodium Nadroparin) was 0.35 ± 0.15 mL/session in the DOLHDF group and 0.5 ± 0.1 mL/session in the OLHDF group ($p < 0.05$).

There was a significant correlation between the C reactive protein level and the post-dialysis GSSG/GSH ratio ($r = 0.65$; $p = 0.02$) (fig. 4).

DISCUSSION

Blood levels of GSSG and the GSSG/GSH ratio are indexes of oxidative stress under different physiological and pathological conditions. Besides, blood gluthathione reflects the gluthathione state in other less accessible tissues.

Patients suffering from end-stage chronic renal disease present decreased blood levels of GSH. This has already been shown in several previous works, although in most of them the determination of GSH is done at the erythrocyte level.[18-20] By contrast, the GSSG level is increased as compared with that in the normal population.

In our patients we did not observe significant differences in GSH levels after the hemodialysis session in any of the study groups. In previous studies there exist some controversy regarding the changes in GSH levels after the hemodialysis session.[21]

About GSSG, we observed a significant increase in the level of oxidized gluthathione after the hemodialysis session. We did not observe significant differences between the groups regarding pre- and post-dialysis GSSG levels.

This increase in oxidized gluthathione levels shows that hemodialysis increases oxidative stress, confirming what has been reported in previous studies.[11, 22, 23]

Similarly, when we analyzed the GSSG/GSH ratio, one of the most sensitive indicators of oxidative stress, we could verify that after the hemodialysis session the ratio was significantly increased with both hemodialysis techniques.[18]

In the DOLHDF group, we observed a tendency to present lower GSSG/GSH ratios, suggesting that oxidative stress in patients submitted to this technique is lower than with OLHDF. The lower PCR values in patients on DOLHDF support this hypothesis.

These findings may be justified by the fact that these patients had better dialysis doses, better clearance of intermediate molecular weight substances, and lower chronic volume overload; all of these factors have been implicated in the chronic inflammatory response found in these patients.

Turi et al. showed that the reduction in the anti-oxidant capacity and GSH content was the result of the accumulation of red blood cells metabolites and ure-

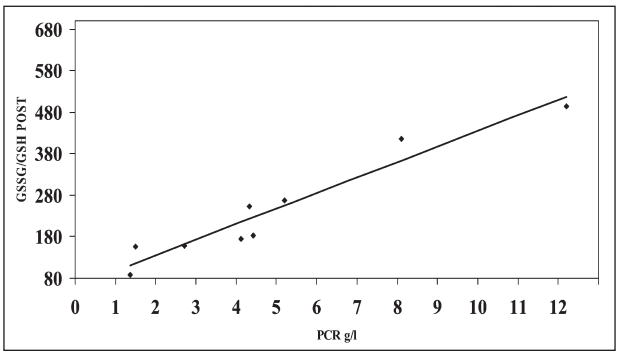

Fig. 4.— Correlation between post-dialysis GSSG/GSH ratio and C reactive protein levels.

dialysis because most of them could not be dialyzed.[24] Our two groups of patients were submitted to on-line hemodiafiltration, although the patients in the group of three weekly sessions received a higher number of convection liters as compared with those with daily sessions.

In previous works, it has been verified that the use of techniques with high convective volume produces an important loss of water-soluble vitamins, among which are vitamin C, one of the main anti-oxidants.[25]

About erythropoietin, the patients in the group of daily hemodialysis received lower EPO doses (2000 IU/week). These lower erythropoietin requirements may be related, as it has been already mentioned, with a lower dialysis dose, but also with lower exposure to oxidative stress observed in this group.

In this sense, Locatelli et al. suggested that supplementing these patients with anti-oxidants might improve their anemia and decrease EPO requirements.[1]

The DOLHDF group received higher dialysis dose (eKR) than the OLHDF group ($p = 0.0009$), and it has been already mentioned, this factor may explain lower erythropoietin requirements, lower GSSG/GSH ratios, and from an inflammatory point of view lower PCR levels. About this, we may observe a significant correlation between PCR levels and the post-dialysis GSSG/GSH ratio, that is to say he higher the PCR the higher the ratio. Generally speaking, we may state that the patients in the DOLHDF group presented lower inflammatory state.

Obviously, the low number of patients included into the study, the fact that the patients were not their own control, and the variability of the population samples are big limitations of the study; in spite of this, this small DOLHDF group showed a trend to present an improvement in very sensitive markers of oxidative stress such as the GSSG/GSH ratio. This fact, together with other largely demonstrated, such as better control of arterial hypertension, anemia, and osteodystrophy, makes us consider another likely advantage of DOLHDF.

The activity of xanthine oxidase, an enzyme generating superoxide anion, is elevated in the plasma and liver of patients with oxidative stress-associated pathologies.

Inhibition of xanthine oxidase has been shown to be effective in order to improve endothelial dysfunction of patients with hypercholesterolemia. Recently, Butler *et al*. have shown that alopurinol protects against endothelial dysfunction in diabetic patients with mild arterial hypertension.[26] It has also been shown that septic patients present activation of xanthine oxidase that is related with increased levels of free radicals.[27] We have not find significant differences in xanthine oxidase levels before and after dialysis, although in both groups of patients we could observe a trend towards a not significant descent when determining the pre- and pos-dialysis activity of this enzyme.

In summary, we may conclude that patients suffering from end-stage chronic renal disease receiving replacement therapy are submitted to additional oxidative stress. Daily-administered on-line hemodiafiltration improves oxidative stress. However, there still is too much work to be done to find a replacement technique as much physiological as possible. It may be that daily hemodialysis, as shown in this work, will be a future option.

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