Oxidative stress evaluation in uraemic patients undergoing continuous ambulatory peritoneal dialysis

G. Mircescu[1], C. Capusa[1], I. Stoian[2], D. Lixandru[2], E. Rus[3], L. Coltan[2] and L. Gaman[2]

[1]"Dr Carol Davila" University Hospital of Nephrology, Bucharest, Romania
[2]Biochemistry Dept. "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
[3]NephroCare "Dr Carol Davila" Dialysis Centre, Bucharest, Romania

Abstract

Since the great bulk of evidences regarding oxidative stress associated with chronic renal failure (CRF) came from haemodialysis patients and only scant data is available concerning peritoneal dialysis, we investigated the oxidative status in CRF patients on continuous ambulatory peritoneal dialysis (CAPD).
Plasma lipid peroxidation assayed as thiobarbituric acid reactive substances (TBARS; nmol/g protein), plasma total free thiols (Pt-SH; mcmol/g protein) as marker of extracellular antioxidant status and parameters of enzymatic/non-enzymatic intracellular antioxidant defence (erythrocyte superoxide dismutase (SOD; U/g haemoglobin), glutathione peroxidase (EGPx; U/g haemoglobin) and non-protein thiols (E-SH; mcmol/g haemoglobin)] were measured by spectrophotometry in 18 CAPD patients, 16 non-dialysed CRF patients and 12 healthy subjects.
Increased TBARS were found only in CAPD patients versus Controls (58.3±19.8 vs. 42.7±12.4, p= 0.01). Also, they had lower activity of erythrocyte SOD (164.2±47.5 vs. 463.2±77.5 in CRF group, p<0.001 and 409.8±58.8 in Controls, p<0.001). Plasma thiols were decreased (6.3±0.9 in CAPD group, p=0.03 and 6.1±1.0 in CRF group, p=0.02 vs. 7.2±1.1), while EGPx and E-SH showed gradually elevated values in patients, with the bigger increment in CAPD group (EGPx: 3.3±0.9 in Controls, 4.5±1.3 in CRF group, p=0.01, and 7.9±0.9 in CAPD group, p<0.001; E-SH: 3.2±0.9 in Controls, 4.8±2.5 in CRF group, p= 0.04, and 11.8±1.8 in CAPD group, p<0.001).
CAPD patients seem exposed to oxidative stress, evidenced by both an increase in plasma lipid peroxidation and a suppressed activity of antioxidants (SOD, Pt-SH). The enhancement of erythrocyte glutathione-dependent antioxidants, as an adaptive response to oxidative processes, indirectly argues in favour of oxidative stress occurring before initiation of dialysis and augmented thereafter.

Key words: chronic renal failure, oxidative stress, peritoneal dialysis

Introduction

Oxidative stress, primarily due to the uraemic state per se, but also to certain aspects of dialysis procedure, has been documented in chronic renal failure (CRF) patients. Markers of oxidative damage like increased lipid peroxidation products, increased plasma protein carbonyl groups and decreased plasma protein thiol groups, as well as reduced antioxidant defence parameters were reported before the initiation of dialysis therapy and seem to gradually worsen as the renal function declines (1).
Dialysis-related factors could add deleterious effects on the oxidant status of CRF patients with regard to both reactive species generation and antioxidant losses. The bioincompatibility of the dialysis materials such as dialyser membranes (especially cellulose) and bacterial contaminants from dialysate were involved in the augmented production of reactive oxygen species in haemodialysis patients. However, their roles are still debated (1).
Also, peritoneal dialysis might enhance oxidative stress by the presence of high concentration of glucose in dialysis fluid (2). Moreover, the glucose degradation products formed mainly during the heat sterilisation process of the solutions, as well as the low pH and the high osmolality contribute to the bioincompatibility of the conventional peritoneal dialysis fluids that could account for the increased local and systemic oxidative burden (3). In addition, impairment of the antioxidant status, expressed by lower levels of plasma ascorbate and whole-blood reduced glutathione was found in chronic peritoneal dialysis patients (3).
Considering that the great bulk of evidences about uraemia-associated oxidative stress came from haemodialysis patients and only scant data is available concerning peritoneal dialysis, we aimed to evaluate the oxidative status in CRF patients on continuous ambulatory peritoneal dialysis (CAPD).

Patients and methods

Subjects

Eighteen clinically stable CAPD patients, on renal replacement therapy (RRT) for at least 9 months (median vintage on RRT 35 months, between 13-110 months), none of them treated with erythropoietin or intravenous iron were included. Peritoneal dialysis was performed by four exchanges daily (three in the daytime and one overnight), with 1.5-2L/exchange of conventional heat-sterilised peritoneal dialysis fluids containing 1.36% glucose. Median residual diuresis was 300mL (between 0-1000mL), and

Correspodence to: C. Capusa, "Dr Carol Davila" University Hospital of Nephrology, 4 Calea Griviței, sect. 1, 010731, Bucharest, Romania; E-mail: ccalexandr@yahoo.com

median volume of ultrafiltration per day was 1000mL (between 300-1800mL).
Sixteen age- and gender-matched non-dialysed CRF patients (mean serum creatinine 4.5±2.7mg/dL) were randomly selected from those attending the Nephrology Clinic for routine periodical examination over a period of six months and composed the control patients group. They were not considered candidates for RRT in the near future.
The CRF groups had a similar distribution according to primary renal disease, with primary chronic glomerulonephritis being the main cause of CRF (Table I). There were no patients with diabetic nephropathy.
Exclusion criteria for the patients were inflammatory and infectious disorders (including exit or tunnel infections and/or peritonitis two months before enrolment), malignancies, diabetes mellitus, active liver diseases, severe anaemia, and medications with antioxidative potentialities (vitamin C or E, folic acid, allopurinolum). None of the patients required blood transfusions in the preceding two months.
Normal control group consisted of twelve matched healthy volunteers with normal routine biochemistry and urine analyses. They did not take any medication or nutritive supplements.
An informed consent was obtained from all participants.

Methods

Blood samples were drawn after overnight fasting into standard vacuum tubes with lithium heparin, were transported on ice, in the dark, and were processed within 30 minutes of collection by centrifugation (3000 *g* for 10 minutes at room temperature).
The plasma aliquots were stored at -70°C until analysed for oxidative stress determinations or used immediately for routine laboratory parameters. Erythrocytes were washed three times with isotonic saline solution and lysed in cold ultrapure water. The red cell stroma was removed by centrifugation (10000 *g* for 15 minutes at 4°C) and the clear supernatant was stored at -70°C until erythrocytes antioxidant parameters were assayed. Reagents and ultra pure water were treated with Chelex 100 in order to bind transitional metals.
Usual biochemistry parameters, C-reactive protein included, were determined by means of routine automated procedures on multiparameter analyser and haemoglobin concentrations were measured by automated spectrophotometric method.
All the oxidative stress parameters were determined by spectrophotometry and the measurements were carried out on triplicate samples.

Plasma thiobarbituric acid reactive substances (TBARS), measured according to the method described by Esterbauer (4), were used to assess plasma lipid peroxidation. Plasma aliquots were treated with butylated hydroxytoluene in order to prevent artifactual oxidation during the assay procedure and mixed with 0.67% thiobarbituric acid (TBA) and 20% trichloroacetic acid. Blank samples from distilled water and the same reagents were prepared. The reaction mixture was heated at 100°C for one hour and then cooled with tap water. The pink TBA-reacting substances adduct was extracted in n-butanol and the absorbance of the organic layer was read at 532nm, after centrifugation. The concentration of lipid peroxidation products was calculated as malondialdehyde (MDA) equivalents using the extinction coefficient for the MDA-TBA complex of 153000 M^{-1} cm^{-1} and expressed as nmol/g protein.

Plasma total free thiols (Pt-SH) assayed by the Ellman's method (5), served as marker of extracellular antioxidant status. Briefly, aliquots of plasma were mixed with 5mM phosphate buffer (pH=8) and 10% sodium dodecil sulphate. Then Ellman's reagent was added and samples were incubated at 37˚C for one hour. The absorbance was read at 412nm against a reagent blank. Results were calculated using a calibration curve with glutathione as standard, and expressed as mcmol/g protein.

Erythrocyte superoxide dismutase activity (SOD) was assessed by analysing the rate of inhibition of pyrogallol auto-oxidation, according to Marklund and Marklund (6). The erythrocyte lysate was treated with a mixture of ethanol/chloroform and was centrifuged for 20 minutes at 6000 *g*. The supernatant fluid was mixed with 0.2mM pyrogallol and TRIS buffer (pH = 8.2), in the presence of oxygen. The rate of change in the absorbance at 420nm was monitored for 3 minutes against the blank sample. SOD activity was expressed as U/g haemoglobin.

Erythrocyte glutathione peroxidase activity (EGPx) was determined indirectly by measuring the rate of consumption of NADPH during the regeneration of reduced glutathione from the oxidised glutathione produced by EGPx in the presence of oxidising substrate (7). The erythrocyte lysate was mixed with TRIS buffer and the enzymatic reaction was initiated by adding 1mM tert-butyl hydroperoxide. The conversion of NADPH to $NADPH^{+}$ was followed by recording the decrease in the absorbance at 340nm for 3 minutes. The enzyme activity was calculated using the molar extinction coefficient for NADPH of 6220 M^{-1} cm^{-1} and was expressed as U/g haemoglobin.

Erythrocyte non-protein thiols (E-SH), which consist mainly of reduced glutathione (GSH), were measured according to the method of Beutler *et al* (8). The erythrocyte lysate was mixed with a protein precipitating solution (1.67 g metaphosphoric acid, 0.2 g disodium ethylenediamine-tetraacetic acid and 30 g sodium chloride in 100 ml distilled water). After 5 minutes, the mixture was filtered and 0.3M sodium phosphate and Ellman's reagent were added. The absorbance was read at 412nm against a reagent blank. The results were calculated using the molar extinction coefficient for GSH of 13600 M^{-1} cm^{-1} and were expressed as mcmol/g haemoglobin.

Statistical analysis

Results were expressed as mean ± standard deviation and were compared using Student's *t* test or ANOVA (for normally distributed variables). Median and interquartile range wase used to express the non-parametric variables.
Standard regression analysis and Pearson *r* correlation coefficient were used to evaluate the relationship between pairs of variables.
A p value < 0.05 was considered significant.

Table 1. Main characteristics of the subjects

Parameter	CAPD Group	CRF non-D group	Controls
Number	18	16	12
Main causes of CRF:			
primary chronic glomerulonephritis (%)	44	38	-
tubulo-interstitial nephritis (%)	28	25	-
vascular nephropathies (%)	22	25	-
polycystic kidney disease (%)	0	12	-
other (%)	6	0	-
Mean age (years)	55.6±16.9	55±15.7	45.5±12.1
Gender ratio (M/F)	8/10	10/6	5/7
Smokers in (%)]	3 (16.7)	3 (18.8)	1 (8.3)
Body mass index (kg/m^2)	‡26.2±3.7	†22.8±3.1	25.6±3.0
Serum iron (mcg/dL)	58.6±28.4	54.8±24.7	73.1±24.7
Serum cholesterol (mg/dL)	225.8±57.0	191.9±55.8	229.5±48.1
Serum albumin (g/dL)	†3.7±0.4	†3.9±0.6	4.6±0.3
Haemoglobin (g/dL)	†9.7±2.3	†9.6±2.3	14.1±1.3
Serum creatinine (mg/dL)	†‡9.4±2.9	†4.5±2.7	0.9±0.1
Serum uric acid (mg/dL)	†6.1±0.8	†6.8±1.5	4.7±1.5

$p<0.05$: † versus Controls, ‡ versus CRF non-dialysed Group

Results

There were no significant differences among groups regarding age, gender, smoking habit, main causes of CRF, serum iron or lipids status (Table 1).

The body mass index (BMI; kg/m^2), as a parameter of nutritional status, was reduced in non-dialysed patients than in the other two groups (22.8±3.1 vs. 25.6±3 in controls, p=0.02 and vs. 26.2±3.7 in CAPD group, p=0.01, respectively), but was similar in patients treated by continuous ambulatory peritoneal dialysis and healthy subjects.

Serum albumin and haemoglobin were decreased in all CRF patients compared to controls, while serum uric acid was increased in the same groups, but without significant differences between dialysed and non-dialysed patients. As expected, serum creatinine was higher in uraemic groups than in normal individuals, and was further elevated in CAPD group versus non-dialysed patients.

All subjects had serum C-reactive protein within the normal range (0-5 mg/dL).

The values of the oxidative stress parameters are listed in Table 2.

Table 2. Values of the oxidative stress parameters in studied groups

Parameter	CAPD group	CRF non-D group	Controls	*p* CAPD vs. non-D	*p* CAPD vs. Controls	*p* non-D vs. Controls
TBARS	†58.3±19.8	51.8±20.5	42.7±12.4	0.36	0.01	0.15
Pt-SH	†6.3±0.9	†6.1±1.0	7.2±1.1	0.51	0.03	0.02
SOD	†‡164.2±47.5	463.2±77.5	409.8±58.8	<0.001	<0.001	0.06
EGPx	†‡7.9±0.9	†4.5±1.3	3.3±0.9	<0.001	<0.001	0.01
E-SH	†‡11.8±1.8	†4.8±2.5	3.2±0.9	<0.001	<0.001	0.04

$p<0.05$: † vs. Controls; ‡ vs. CRF non-dialysed Group

Plasma TBARS levels were increased only in CAPD patients versus controls. These patients, also, had markedly lower activity of erythrocyte SOD as compared to the other studied groups. As concerns the glutathione-dependent antioxidants, a significant decrease of plasma thiols was noticed in both uraemic groups, while the EGPx and E-SH showed gradually elevated values in patients, with the bigger increment in CAPD group.

Discussion

There is strong evidence that the pathogenesis of oxidative stress in chronic renal failure patients is multifactorial, and exists long before the initiation of maintenance dialysis treatment (9). However, dialysis procedures have various implications on oxidative processes including reactive species generation due to tissue contact to more or less bioincompatible materials, water-soluble antioxidant losses, as well as removal of some uraemic toxins and oxidising substances (9). The former two effects, leading to an imbalance between oxidants production and antioxidant defence mechanisms, might impose additional oxidative stress on uraemic patients.

In accordance with other studies (10,11), we found a significant increase of the lipid peroxidation marker in plasma from CAPD patients compared to normal controls, indirectly suggesting an enhancement of reactive oxygen species generation in uraemic patients on peritoneal dialysis. A tendency toward higher TBARS levels was also seen in CRF non-dialysed group, but without reaching statistical significance. This might argue against significant oxidative processes accounting for lipid peroxidation in CRF before

renal replacement therapy, but more probably it is related to the low sensibility and specificity of the used assay. Alternatively, the serum levels of TBARS might be kept in the normal range by some protective mechanisms like hepatic microsomes that are capable of both TBARS generation and degradation, as supported by the study of *Gotoh* et al (12).

Previously, *Malgorzewicz* et al also discovered higher plasma content of MDA in peritoneal dialysis compared to healthy group, especially in severely malnourished patients and supposed that the increase in oxidative stress may be related to a degree of malnutrition (13). Our findings support the presence of oxidative stress in uraemic patients on CAPD with no apparent deficiency of the nutritional status, at least as reflected by the BMI and serum cholesterol (that did not differ from Controls), or serum albumin (that was similar to CRF group). Furthermore, the CRF group who had lower BMI and serum albumin than normal controls showed no differences in TBARS levels compared to healthy subjects. However, the present study does not provide enough data to sustain or deny the role of malnutrition in augmenting oxidative stress because the evaluation of nutritional status was not a primary objective and its casual assessment relied on few, unspecific parameters.

Indirect evidences of oxidative stress can also be obtained from the investigation of antioxidant parameters. The decrease of plasma total free thiols levels observed in all studied CRF patients compared to healthy individuals, which reflects a deficiency of the extracellular antioxidant defence, could be speculated in favour of an increased consumption for counteracting the potential oxidative burden associated with chronic renal failure. The presumable contribution of hypoalbuminaemia to the lower plasma thiols appears implausible in our patients since no significant correlation was found between serum albumin and Pt-SH in either CRF groups (r = 0.27, confidence interval 95% = -0.3 ÷ +0.7; p = 0.4 in CRF non-dialysed group, and r = -0.13, confidence interval 95% = -0.47 ÷ +0.32; p = 0.5 in CAPD group, respectively). Therefore, we interpreted the reduced plasma thiols as an indirect proof of augmented oxidative stress in CRF patients, dialysed or not. Similar results have been reported by *Tarng* et al, who demonstrated the decrease of the whole-blood reduced glutathione as well as of the plasma ascorbate and alfa-tocopherol in chronic peritoneal dialysis patients and in non-dialysed patients with advanced renal failure (mean creatinine clearance 11.8±4.6mL/min) (3).

The deficiency of antioxidant systems in peritoneal dialysis patients is further sustained in our results by the marked decline of erythrocyte SOD activity. Therefore, one can assume that both extracellular and enzymatic intracellular defence against oxidative stress are defective in uraemic patients undergoing CAPD. These findings are in agreement with some other studies that reported reduced enzymatic erythrocyte defence (superoxide dismutase and catalase) in CAPD group (14,15), although discrepant results were also obtained. For example, *Bonnefont-Rousselot* et al did not detect any abnormalities in erythrocyte SOD activity in CAPD-treated patients in comparison with age-matched control group (16). However, they included older subjects than ours and this might allow to speculate the lack of difference on the account of advanced age, which impairs the antioxidant systems by itself and, possible, uraemia or dialysis procedure were not able to add further deterioration in this distinctive age group.

Concerning the other two studied erythrocyte antioxidant parameters, contrary to previous published data that described unchanged EGPx and reduced erythrocyte glutathione (11), significantly higher values were noticed in both CRF groups. We hypothesised that an enzymatic induction occurs in response to oxidative stress and accounts for the increased EGPx activity and the regeneration of the intracellular pool of reduced glutathione (GSH). Indeed, the ability of glutathione peroxidase to adapt its activity according to oxidative stress was demonstrated in chronic haemodialysis children (17) and was supposed by *Canestrari* et al in order to explain the increased EGPx found in CAPD subjects (cited by (16)). Also, it was stated that the oxidants-induced up-regulation of glucose-6-phosphate dehydrogenase (G6PD) might play a role in the increase of intracellular GSH. In experimental setting, on culture of human cells, the authors showed that G6PD expression is rapidly enhanced in response to oxidative stress and it controls intracellular reduced glutathione without interfering with the activity of the other enzymes involved in the peroxide/hydroperoxide-detoxifying pathway (18). As a result, cells overexpressing G6PD had an overall reduced state, although the precise biochemical mechanism underlying the enzyme's antioxidant properties is still debated. Until now, two alternative explanations were proposed: either G6PD influences the output of GSH and thus contribute to the maintaining of an intracellular redox potential, or it is implicated in a mechanism that promotes glutathione storage (18). Anyway, these experimental inferences support our interpretation of the increased EGPx and E-SH in uraemic patients as an adaptive reaction to enhanced oxidative stress.

Conclusions

We concluded that peritoneal dialysis patients are exposed to oxidative stress, evidenced by both an increase in plasma lipid peroxidation (TBARS) and a suppressed activity of antioxidants (erythrocyte SOD). Also, the impairment of extracellular antioxidant system expressed by the lower levels of plasma free thiols, probably accounted for by the enhanced consumption in order to offset the oxidative burden, plead for an imbalance between reactive species production and antioxidant defence mechanisms in CAPD patients. The enhancement of erythrocyte glutathione-dependent antioxidants might reflect rather an adaptive response to oxidative processes and, therefore, indirectly argues in favour of oxidative stress occurring before initiation of dialysis and augmented thereafter.

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