Original Article

The association of advanced glycation end-products with glutathione status

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Abstract

Background: The aim of this study was to investigate oxidative stress with regard to the concentrations of advanced oxidation protein products (AOPP), advanced glycation end-products (AGEs), pentosidine, glycated albumin, reduced glutathione (GSH) and oxidized glutathione (GSSG), glutathione redox ratios and thiobarbituric acid-reactive substances (TBARS) in non-diabetic patients undergoing continuous ambulatory peritoneal dialysis (CAPD).

Methods: The study group consisted of 52 non-diabetic CAPD patients and 34 healthy controls. AOPP, AGEs, pentosidine and glycated albumin were measured in plasma, whereas GSH, GSSG and TBARS concentrations were measured in erythrocytes of both patients and controls.

Results: All parameters were found to be significantly increased, except the glutathione redox ratio, which was found to be decreased in patients undergoing CAPD. Multiple regression analysis showed that AGEs were the only independent predictor of glutathione redox ratio, whereas AGEs, glycated albumin and TBARS were each found to be independent predictors of albumin concentration.

Conclusion: Our results support the hypothesis that oxidative stress and AOPPs/AGEs constitute important risk factors in CAPD patients. The negative relationship between albumin and both AGEs and TBARS suggests that the decrease in albumin may contribute to the increased advanced glycation and lipid peroxidation. The negative relationship between glutathione redox ratio and AGEs suggests that late products of glycation play an important role in the development of oxidative stress observed in patients undergoing peritoneal dialysis treatment.

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Introduction

Patients with chronic renal failure (CRF), especially those receiving regular dialysis treatment, are at high-risk for oxidative damage caused by free radicals, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), superoxide anion radical ($O_2^{-\bullet}$) and nitric oxide.[1] It is known that polymorphonuclear leukocytes from patients with CRF produce increased amounts of reactive oxygen species in the resting state as evaluated by chemiluminescence[2] and these reactive species can diffuse outside from the phagocytic cells and interact with red blood cells (RBCs).[3] In previous studies, oxidative stress has been examined in patients with end-stage renal disease (ESRD) undergoing haemodialysis and continuous ambulatory peritoneal dialysis (CAPD).[4,5] During oxidative stress, modification of proteins can occur resulting in the formation of advanced oxidation protein products (AOPP) and advanced glycation end-products (AGE) in ESRD.[6]

AGE[7] and AOPP[8,9] are a class of uraemic toxins that occur during kidney failure and haemodialysis treatment. It is known that plasma AOPP concentration may be a useful marker of protein oxidative damage, measuring highly oxidized proteins and especially albumin.[8] In addition, the increased oxidative stress enhances the formation of AGE precursors along with an impaired clearance and may cause their accumulation in renal failure. These processes may further result in the generation and accumulation of AGE. Serum AGE circulate in the form of modified proteins or peptides and, in the case of pentosidine, also in their free form.[10]

Glycated albumin is a type of albumin which is modified by Amadori glucose adducts and several studies have established that exposure of renal glomerular cells to non-enzymatically glycated albumin induces alterations in biosynthetic and signalling pathways that are involved in the pathogenesis of diabetic nephropathy.[11,12] Lamb *et al.*[13] have found increased glycated albumin concentration in serum and dialysate of patients undergoing CAPD.

Reduced glutathione (GSH) is the most important and frequently investigated scavenger of reactive oxygen species in RBCs of patients on dialysis.[14] Glutathione is a sulphydryl tripeptide (γ-glutamyl-cysteinyl-glycine) that acts as an antioxidant, as antitoxin and as an enzyme co-factor.[15] It is present in cells as GSH, the predominant form, and as oxidized glutathione (GSSG), which together attain millimolar concentrations within cells, making this peptide one of the most highly concentrated intracellular antioxidants. Glutathione is transported out of specific cells, such as erythrocytes, either as GSSG or GSH-conjugates and this transport accounts for most of the GSH turnover in these cells.[15]

There is little or no data available that explains the alterations in AOPP and AGEs concentrations, glutathione redox ratio and erythrocyte susceptibility to oxidative stress observed in patients undergoing peritoneal dialysis. In addition, we are unaware of any study of the relationship between glutathione redox ratio and AGE/AOPP in patients receiving CAPD. The present study was therefore undertaken to investigate the changes in glutathione redox ratios, AGE, AOPP and lipid peroxidation concentrations in non-diabetic CAPD patients and their associations with one another.

Methods

Subjects

One-hundred and twenty non-diabetic patients undergoing peritoneal dialysis for at least three months at the Akdeniz University School of Medicine and at Antalya State Hospital were screened for this cross-sectional study. Any patients with evidence of atherosclerotic disease, abnormal electrocardiogram and those with an active infection or a history of peritonitis in the last three months, or who were smokers were excluded from the study. On this basis, a total of 68 patients were therefore excluded from the study.

The final study group consisted of 52 non-diabetic patients undergoing peritoneal dialysis (ages 45.20 ± 2.13 years [mean $\pm$ SEM] and M/F ratio = 25/27), and 34 healthy controls (ages 43.25 ± 2.77 years [mean $\pm$ SEM] and M/F ratio = 16/18).

All of the controls were non-smokers, abstained from alcohol and were not on any medication. The aetiologies of CRF were: hypertension ($n = 22$), chronic glomerulonephritis ($n = 15$), tubulointerstitial nephritis ($n = 10$) and unknown ($n = 5$). During the study, none of the patients received antibiotics, corticosteroids, cytotoxic drugs or any vitamins. In addition, none received parenteral iron prior to or throughout the study. Twenty-two patients had been receiving one or more antihypertensive drugs (calcium channel blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, oral nitrates or beta blockers) at the time of the study. Twenty-six patients were receiving recombinant human erythropoietin therapy and the mean dosage was 52 ± 27 IU/kg body weight/week. Twenty patients were on statin treatment for hyperlipidaemia. All patients undergoing CAPD were performing 4 h, 2 L exchanges per day with standard acidic, lactate-based glucose dialysis solution. They were on the Baxter Twin Bag system or the Fresenius Stay Safe system. Dwell times were generally 4–6 h during the day and 8 h overnight. The glucose concentration ranged from 1.36% to 3.86%. A peritoneal equilibration test was also performed in each patient as described elsewhere.[16] According to this, the patients had neither a highly permeable peritoneal membrane nor had been using icodextrin dialysis solution.

Dialysis adequacy was assessed by measuring Kt/V (total Kt/V: median, 2.02 [interquartile range, 1.82–2.49]; renal Kt/V: median, 0.18 [interquartile range, 0.00–0.70]).

This study was approved by the Ethics Committee of Faculty of Medicine of Akdeniz University, Antalya, Turkey and all participants gave written consent.

Blood collection

Blood samples were taken after an overnight fast and were collected in 5 mL plain and 10 mL Vacutainer® heparinized tubes (BD Vacutainer Systems, Plymouth, UK). The blood samples were centrifuged at 3000 *g* for 10 min. Routine blood counts and blood chemistry were analysed by standard laboratory procedures in serum samples. Serum glucose, uric acid, total cholesterol, triglyceride, albumin and haemoglobin concentrations were assayed using standard methods on a Modular P automated analyser (Roche Modular Diagnostics, Basel, Switzerland). HDL cholesterol concentrations were measured using a direct method, also on the same analyser, and LDL cholesterol concentrations were calculated using the Friedwald equation ([LDL-chol] = [total chol] – [HDL-chol] – [triglyceride]/2.22).[17] C-reactive proteins (CRP) were measured by immunoturbidimetry and parathyroid hormone (PTH) by electrochemiluminescence (ECLIA) on Roche Modular E170 analyser.

Plasma samples were collected and stored at -70° until assayed. Plasma was used for the estimation of AOPP, AGE, pentosidine and glycated albumin.

The remaining RBCs were washed three times with an ice-cold isotonic sodium chloride solution (1:10, v/v) and the obtained packed cells were resuspended in the washing solution to give a 50% suspension. Haemolysis of the washed cell suspension was achieved by mixing one volume of cells with nine volumes of cold distilled water. Concentration of thiobarbituric acid-reactive substances (TBARS) was measured in a fresh suspension that contained 1 g/100 mL of haemoglobin. Some of the whole blood was used immediately to determine GSH and GSSG concentrations. All assays were carried out in duplicates. All of these operations were performed at 4°C.

Determination of advanced oxidation protein products

Determination of AOPP was based on spectrophotometric detection as described by Kalousova *et al.*[6] and Witko-Sarsat *et al.*[8] Two-hundred microlitres of serum, diluted 1:5 with phosphate-buffered saline (PBS) pH 7.4, 200 μL of chloramin T (0–100 μmol/L as calibrator) and 200 μL of PBS as blank were applied on a microtiter plate. Ten microlitres of 1.16 mol/L KI and 20 μL of acetic acid

were added and the absorbance at 340 nm measured immediately. The concentration of AOPP was expressed in the amount of chloramines T per gram of protein (μmol/g protein).

Determination of advanced glycation end-products

Determination of AGE is based on spectrofluorometric (Shimadzu RF-5000, Kyoto, Japan) detection according to Henle *et al.*[18] Plasma samples were centrifuged at 25,000 *g* for 60 min at 4°C. The plasma was then diluted 1:50 (v/v) with PBS pH 7.4, and the fluorescence intensity emitted at a wavelength of approximately 440 nm was recorded, following excitation at 350 nm, using a Shimadzu RF-5000 spectrofluorometer (Shimadzu). The fluorescence intensity was expressed in arbitrary units per gram of protein (AU/g protein).

Determination of pentosidine

Determination of pentosidine is based on spectrofluorometric detection according to the procedure of Gugliucci and Menini.[19] Plasma samples were centrifuged at 25,000 *g* for 60 min at 4°C. The plasma was then diluted 1:50 with PBS pH 7.4, and the fluorescence intensity emitted at a wavelength of approximately 385 nm was recorded, following excitation at 335 nm, using a Shimadzu RF-5000 spectrofluorometer (Shimadzu). Fluorescence intensity is expressed in arbitrary units per gram of protein (AU/g protein).

Determination of glycated albumin

Glycated albumin was determined by using the Glycabumin ELISA kit (Exocell Inc., Philadelphia, PA, USA). A 50 μL plasma sample was added to a tube precoated with an anti-glycated monoclonal antibody and allowed to incubate for 30 min. Glycated albumin was quantified after incubation with an enzyme-conjugated secondary antibody against human serum albumin. Glycated albumin values were expressed relative to total albumin content after determination of total plasma albumin (% glycated albumin = [glycated albumin/total albumin] $\times$ 100).

Determination of whole blood reduced and oxidized glutathione

GSH concentration was assayed by the method of Fairbanks and Klee.[20] GSSG was determined, in haemolysate previously incubated with 4 vinyl-pyridine for 60 min at room temperature, by the method of Tietze.[21] This involved monitoring the changes in absorbance at 412 nm in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid). GSH and GSSG values were expressed as micromoles per gram of haemoglobin.

Determination of thiobarbituric acid-reactive substances

The lipid peroxidation contents of erythrocytes were determined by malondialdehyde production and assayed as TBARS using the method of Stocks and Dormany[22] and the results were expressed as nanomoles per gram of haemoglobin using 1,1,3,3-tetraethoxypropane as standard.

Determination of haemoglobin

Haemoglobin concentrations in whole blood and in RBC haemolysate were determined using the cyanomethaemoglobin method of Fairbanks and Klee.[20]

Determination of protein

Protein concentrations were measured by the method of Lowry *et al.*[23] using bovine serum albumin as standard.

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). The data were analysed using the statistical package program SPSS for Windows (Version 13.0; SPSS, Chicago, IL, USA). We used tests of normality to determine if the data was normally distributed. Comparison of parameters between the two groups was performed using Student's *t*-test. When the data distribution was non-Gaussian, non-parametric tests were used. Pearson correlation analysis and multiple stepwise regression analysis were used to determine independent predictors of glutathione redox ratio and albumin. P values <0.05 were considered statistically significant.

Results

Haemodynamic and biochemical parameters of patients and controls are shown in Table 1. Age, gender, systolic and mean arterial blood pressure parameters, total cholesterol, HDL- and LDL-cholesterol concentrations showed no difference between patients undergoing CAPD and controls (Table 1). In patients receiving CAPD, haemoglobin and albumin concentrations were lower than in controls, but glucose, triglyceride, PTH, sCRP and uric acid concentrations were higher (Table 1).

Table 2 illustrates plasma AOPP, AGE, pentosidine, glycated albumin and erythrocyte GSH, GSSG, GSH/GSSG, glutathione redox ratio (GSH/[GSH + 2GSSG]) and TBARS concentrations in CAPD patients and controls.

Correlation analysis showed that there was a negative correlation between albumin and TBARS concentrations ($r = -0.318$, $P < 0.05$) and between glutathione redox ratio and uric acid concentrations ($r = -0.316$, $P < 0.05$). In addition, there was a positive correlation between AOPP and PTH concentrations ($r = 0.380$, $P < 0.05$) in patients undergoing CAPD.

Multiple regression analysis of variables against glutathione redox ratio and albumin concentrations in patients receiving CAPD is shown in Table 3. Age, TBARS, AGE, AOPP, pentosidine, glycated albumin and GSSG were included in a model for the determination of independent predictors of glutathione redox ratio. AGE was found to

Table 1 Haemodynamic and biochemical parameters of CAPD patients and control subjects

Parameters	CAPD patients	Controls	*P* value
Subjects (*n*)	52	34	
Age (years)	45.20 ± 2.13	43.25 ± 2.77	NS
Gender (male/female)	25/27	16/18	NS
Dialysis duration (months)	37.38 ± 4.60		
SBP (mmHg)	122.26 ± 3.65	115.83 ± 2.27	NS
DBP (mmHg)	79.40 ± 2.36	73.86 ± 1.56	<0.05
MAP (mmHg)	94.57 ± 2.68	99.79 ± 2.33	NS
Haemoglobin (g/L)	107.8 ± 4.70	140.8 ± 3.00	<0.001
Glucose (mmol/L)	5.44 ± 0.21	4.75 ± 0.16	<0.05
Albumin (g/L)	39.7 ± 0.90	45.9 ± 1.00	<0.001
Total cholesterol (mmol/L)	4.94 ± 0.19	4.83 ± 0.20	NS
HDL-cholesterol (mmol/L)	1.40 ± 0.14	1.33 ± 0.06	NS
LDL-cholesterol (mmol/L)	2.76 ± 0.15	2.92 ± 0.16	NS
Triglycerides (mmol/L)	1.81 ± 0.15	1.30 ± 0.13	<0.01
Parathyroid hormone (ng/L)	0.34 ± 0.07	0.04 ± 0.00	<0.001
sCRP (mg/L)	7.90 ± 1.50	2.00 ± 0.30	<0.001
Uric acid (mg/L)	57.70 ± 2.50	46.40 ± 2.30	<0.01

Values are expressed as means ± SEM. *P*, significance value vs. controls. NS, not significant. CAPD, continuous ambulatory peritoneal dialysis; DBP, diastolic blood pressure; MAP, mean arterial pressure; SBP, systolic blood pressure; sCRP, sensitive C-reactive protein

be the only independent predictor of glutathione redox ratio in these patients ($P < 0.01$). In contrast, AGE ($P < 0.05$), glycated albumin ($P < 0.05$) and TBARS ($P < 0.05$) were each found to be independent predictors of albumin concentration in these patients.

Discussion

It has long been known that CRF is associated with oxidative stress and that this stress is exacerbated by dialysis treatment.[24]

Although several studies have been made on the role of AOPP and AGE in renal disease,[7–9] these parameters have not been well characterized in non-diabetic patients with renal disease. In our study, we found significantly increased concentrations of glycated albumin, AGE and AOPP in patients undergoing CAPD. Witko-Sarsat *et al.*[8] proposed the measurement of AOPP as a reliable marker to estimate the degree of oxidant-mediated protein damage in uraemic patients and to predict the potential efficacy of therapeutic strategies aimed at reducing such an oxidative stress. At the biochemical level, it has been reported that reactive oxygen species act directly on proteins producing AOPP and on sugars (or lipid or amino acids) producing carbonyl groups.

Table 2 Parameters in continuous ambulatory peritoneal dialysis (CAPD) patients and control subjects

Parameters	CAPD patients	Controls	*P* value
Subjects (*n*)	52	34	
AOPP (μmol/g protein)	3.34 ± 0.07	2.00 ± 0.06	<0.001
AGEs (AU/g protein)	201.76 ± 0.96	64.30 ± 0.84	<0.001
Pentosidine (AU/g protein)	253.84 ± 1.23	75.34 ± 1.52	<0.001
Glycated albumin (GlyAlb%)	0.92 ± 0.04	0.73 ± 0.03	<0.01
GSH (μmol/g Hb)	3.93 ± 0.10	3.37 ± 0.12	<0.01
GSSG (μmol/g Hb)	0.23 ± 0.01	0.13 ± 0.01	<0.001
GSH/GSSG ratio	17.76 ± 0.49	27.79 ± 1.28	<0.001
GSH/(GSH + 2GSSG)	0.90 ± 0.00	0.93 ± 0.00	<0.001
TBARS (nmol/g Hb)	25.19 ± 0.11	3.35 ± 0.12	<0.001

Values are expressed as means ± SEM. *P*, significance value vs. controls. AGEs, advanced glycation end products; AOPP, advanced oxidation protein products; GlyAlb, glycated albumin; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH/(GSH + 2GSSG), glutathione redox ratio; TBARS, thiobarbituric acid-reactive substances; Hb, haemoglobin

Table 3 Multiple regression analysis of variables against glutathione redox ratio and albumin in continuous ambulatory peritoneal dialysis patients

Dependent variable	Independent predictors	β	*t*-Test value	*P* value
Glutathione redox ratio	AGEs	−0.334	−2.869	<0.01
Albumin	AGEs	−0.312	−2.178	<0.05
	Glycated albumin	−0.329	−2.367	<0.05
	TBARS	−0.312	−2.301	<0.05

β, standardized regression coefficient; AGEs, advanced glycation end-products; TBARS, thiobarbituric acid-reactive substances

AGE are formed during the non-enzymatic reaction of sugars with proteins. It has been reported that conventional peritoneal dialysis fluids may facilitate AGE formation and increase auto-oxidation by increasing oxidative factors and reducing the antioxidant response by the polyol pathway.[25] This is thought to be due to the high glucose concentration or the presence of reactive glucose degradation products, which are formed during heat sterilization of peritoneal dialysis fluids.[26] The increase in concentrations of AGE, independent of hyperglycaemia, is thought to be a result of oxidative stress, or dyslipidaemia and/or decreased renal clearance of AGE.[27]

Because GSH can directly scavenge free radicals and is able to exist in the centre of several enzymatic reactions (because of it being the substrate of glutathione peroxidase and glutathione reductase), we evaluated the ability of RBCs to inhibit oxidative stress, by measuring GSH/GSSG. We found elevated concentrations of GSH in patients receiving CAPD, a finding supported by several studies,[28–31] although conflicting results have been reported.[32,33] The increase in GSH concentration may indicate that RBC develops an adaptive mechanism against oxidative stress by adjusting activities of antioxidant enzymes that are involved in the production of GSH and GSSG. GSSG concentrations in patients undergoing CAPD were found to be higher than those observed in healthy controls. This finding also supports the idea that CRF is characterized by increased oxidative stress.

It has been shown that the molar glutathione redox ratio (GSH/[GSH + 2GSSG]) is a more valuable marker in dialyzed and predialysis patients.[34,35] In our study, the glutathione redox ratio observed in patients receiving CAPD was found to be 64% of that observed in controls. This finding is supported by the study of Canestrari *et al.*,[28]

who observed a glutathione redox ratio in RBCs and plasma of patients undergoing CAPD to be 50% of that found in controls. One possible explanation for this observed decrease in the glutathione redox ratio in patients undergoing CAPD may be the increased GSSG concentrations observed in these patients.

The original purpose of our study was to observe the close relationship between AGE concentrations and glutathione redox ratios in patients undergoing CAPD using multiple regression analysis. The strong relationship between these parameters may primarily be related to the increased GSSG concentrations. In a similar way, Deuther-Conrad *et al.*[36] reported that H_2O_2 might be the mediator of the chronic AGE-induced increase in GSSG, as it is produced e.g. by the chemical reaction of AGE precursors, the AGE-induced activation of nicotinamide adenine dinucleotide phosphate oxidase or through disturbance of mitochondrial respiration. In their study, human neuroblastoma cells without AGE maintained a low concentration of GSSG, whereas GSSG concentrations increased to about 4%, 10% and 12% during incubation with 50, 100 or 150 mmol/L bovine serum albumin (BSA)-AGE for 24 h, respectively. Our results lead us to believe that the increase in late glycation of proteins might be related to the increased GSSG concentrations observed in non-diabetic patients undergoing CAPD. However, the findings of our study do not prove that AGE act on glutathione directly in these patients. Further work is needed to clarify the relationship between glycation and glutathione status at the molecular level in these patients. We also found a negative correlation between glutathione redox ratio and uric acid concentrations ($r = -0.316$, $P < 0.05$) in patients undergoing CAPD. It has been reported that an increased uric acid concentration is associated with an increased production of oxygen free radicals.[37] In a study to evaluate oxidative stress in patients with CRF, Erdogan *et al.*[38] found high concentrations of uric acid in these patients, and reported that the high uric acid concentrations found in patients undergoing peritoneal and haemodialysis might be partly responsible for the increase in total antioxidant activity values. Our result indicates that the elevated concentrations of uric acid might affect antioxidant defence system through the glutathione pathway.

We found increased TBARS concentrations in erythrocytes of patients undergoing CAPD, findings supported by those of Canestrari *et al.*[28] and Ozden *et al.*[33] who found increased TBARS concentrations in both erythrocytes and plasma of these patients. We found a weak negative correlation between albumin and TBARS concentrations ($r = -0.318$, $P < 0.05$). We believe one explanation for this could be that albumin concentrations due to it being both a negative acute phase protein and also a recognized antioxidant.[39]

In conclusion, this study has shown that patients undergoing CAPD have an increased susceptibility to oxidative stress. It has also highlighted a strong correlation between glutathione redox ratio and AGE concentration in these patients. Further studies are needed to examine the relationship between AGE/AOPP and glutathione redox status, and the mechanisms behind the changes in oxidative stress observed in patients undergoing CAPD.

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