Evaluation of advanced glycation end products and carbonyl compounds in patients with different conditions of oxidative stress

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Advanced glycation end products (AGE) and dicarbonyl compounds accumulate in serum and tissues of patients with diabetes and chronic renal failure. Pentosidine, free pentosidine, glyoxal and methylglyoxal have been evaluated in plasma of diabetic patients with poor metabolic control at baseline and after the improvement of glycemic levels, and in plasma and peritoneal dialysate of patients with renal failure before and after 12 h of peritoneal dialysis. In diabetic patients, acceptable metabolic control was unable to normalize levels of pentosidine (after 2 and 10 months), glyoxal and methylglyoxal (after 2 months). In patients with end-stage renal disease, mean values of pentosidine, free pentosidine, glyoxal and methylglyoxal decreased in plasma after dialysis. No pentosidine or free pentosidine were present in the peritoneal dialysate at time 0, but were found after 12 h of peritoneal dialysis; glyoxal and methylglyoxal decreased after 12 h of dialysis. So, glyoxal and methylglyoxal, already present in the dialysis fluid, can react with the peritoneal matrix protein, giving a reason for the gradual loss of peritoneal membrane function often observed in patients undergoing long-term peritoneal dialysis.

Keywords: Carbonyl compounds / Diabetes / End-stage renal disease / Metabolic parameters / Non-enzymatic glycation / Peritoneal dialysis

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1 Introduction

The process of non-enzymatic glycation of proteins [1] plays an important role in the pathogenesis of chronic complications associated with diabetes and renal failure. The products of this reaction, such as advanced glycation end products (AGE) and dicarbonyl compounds (glyoxal and methylglyoxal), can cause structural and functional changes in tissue [2–6]. Recent evidence shows that chronic hyperglycemia leads to tissue injury through a common pathway: increased oxidative stress [7]. In renal failure, AGE accumulate as a result of decreased excretion and increased generation caused by oxidative and carbonyl stress of uremia [4–6, 8, 9].

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Abbreviations: AGE, advanced glycation end products; **PD**, peritoneal dialysis

A series of AGE have been structurally quantified, one of which, pentosidine, a glyco-oxidation product and fluorescent crosslink, has been reported as a prognostic marker of the chronic complications of diabetes and end-stage renal disease [5, 6, 8–10].

Approximately 95% of pentosidine in plasma is linked to proteins, with 5% present in its free form. Free pentosidine, due to its very low concentrations, cannot be detected in either normal or diabetic subjects with normal renal function, as it is accumulated by decreased glomerular filtration [5].

Pentosidine could be a useful index of long-term metabolic control in diabetes, however, few studies have been published on the effect of metabolic control of AGE in diabetic patients [11–14]. HbA1c, an early glycation product, can be used to evaluate metabolic control in diabetics because it is related to the plasma level of glycaemia during the 4 to 6 wk preceding the exam [15].

Plasma levels of free pentosidine may provide a useful marker for the plasma levels of AGE in patients with end-

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stage renal disease [16]. Studies on diabetic rats have shown high levels of glyoxal and methylglyoxal in the kidney, lens and plasma, and they increase proportionally to the glucose concentration [4, 17]. It has also been shown in patients with end-stage renal disease that not only AGE but also dicarbonyl compounds form during heat sterilization of glucose-based peritoneal dialysis fluids [18, 19].

So we think it is of interest to evaluate pentosidine, glyoxal, and methylglyoxal in diabetic patients with different levels of metabolic control and to compare them with the standard metabolic control parameters, such as plasma glucose and HbA1c.

We also measured these compounds and free pentosidine in non-diabetic patients with end-stage renal disease to evaluate the impact of PD fluid on AGE and dicarbonyl compounds.

2 Materials and methods

2.1 Subjects

Ten poorly controlled type 2 diabetic patients with normal renal function (plasma creatinine 0.9 ± 0.2 mg/dL; mean ±SD), (M = 6; F = 4), mean age 56 ± 6 years, with a mean disease duration of 2.3 ± 0.6 years, were evaluated at baseline and after achieving good metabolic control. No patients showed evidence of chronic diabetic complications and they were all normotensive. They took no antihypertensive drugs. All patients were treated with intensified insulin therapy (multiple injection of short-acting insulin before each meal and intermediate/acting insulin at bedtime) to rapidly improve their glycemic state, verified on the basis of plasma glucose and HbA1c. Patients were closely monitored during the study: they attended our clinic monthly for 12 months and checked fasting and post-prandial plasma glucose levels at home. The second evaluation of patients was done after 2 months, then they were re-evaluated for a third time after 10 months: glyoxal and methylglyoxal were not measured on that occasion.

Eight non-diabetic patients with end-stage renal disease were examined at baseline and after 12 h peritoneal dwell time. The patients in the study had a mean age (±SD) of 61 ± 9 years, a mean duration of dialysis treatment of 23 ± 26 months, a mean duration of chronic renal disease of 103 ± 63 months. The biochemical parameters of these patients showed mean values of 8.5 mg/dL ± 1.5 of creatinine, 7 mg/dL ± 0.45 of plasma proteins and 92 ± 5 mg/dL of fasting plasma glucose.

Both diabetic patients and those with end-stage renal disease were instructed to consume an isocaloric diet consisting of 16% energy from proteins, 29% from lipids and 55% from complex carbohydrates. This dietary regimen remained unmodified during the study period.

The control group was composed of 20 healthy subjects (10 males, 10 females), with normal glucose tolerance according to American Diabetes Association criteria [20], and normal renal function (plasma creatinine 0.8 ± 0.2 mg/dL), and mean age 53 ± 4 years. Healthy subjects followed an isocaloric diet with lipids below 30% and rich in complex carbohydrates (50 to 55%).

Fasting blood samples were taken on study days to determine plasma glucose, HbA1c, pentosidine, glyoxal and methylglyoxal in diabetic and normal subjects.

Peritoneal dialysis was performed by four exchanges daily with 2-L bags of PD solutions containing varying glucose concentrations (Dianeal, Baxter, Healthcare, S.A. Ireland). The study was done using a 2.27% Dianeal-Baxter glucose solution for a 12-h peritoneal dwell time.

Plasma samples were collected at time 0 and after 12 h dwell time. Samples of PD solutions were collected at time 0 from bags (before infusion into the peritoneal cavity), and samples of dialysate were collected after a 12-h dwell time in patients with end-stage renal disease.

The study was done in accordance with the Declaration of Helsinki: informed written consent was obtained from all subjects participating in the study, and approval was granted by the local ethics committee.

2.2 Methods

Plasma glucose was measured by a glucose-oxidase method [21]. HbA1c was quantified by an LC method [22] (Bio-Rad Laboratories, Milan, Italy). Pentosidine was measured with an LC method [23]. Plasma samples (350 µL), after the addition of 100 µL of NaBH4 (0.2 M), were precipitated on ice with an equal volume of 10% cold TCATCA. The pellet was hydrolyzed in 6 N HCl at 110°C for 18 h. Acid was removed by vacuum centrifugation (Speed Vac SC 110, Savant Instruments Farmingdale, NY, USA). Hydrolyzed pellets were suspended in 500 µL of 0.01% HPTF (heptafluorobutyric acid, Sigma Aldrich, Milan, Italy) and then filtered through 0.45-µm membrane filters (Minisart, Sartorius). Samples were analyzed by LC (Prostar, Varian, Turin, Italy). The column was a 3.9 × 300 mm C-18 µ-bondapack (Waters Italia, Milan, Italy). The LC equipment was programmed with a linear gradient from 0 to 39 min, from 10 to 17% of ACNACN in water and 0.1% heptafluorobutyric acid. Pentosidine was detected by fluorescence excitation at 335 nm and emission at 385 nm. Pentosidine standard was a kind gift from Prof. V. M. Monnier.

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Free pentosidine, evaluated by the LC method [16], was expressed as pmol/mL plasma or dialysate, respectively.

Glyoxal and methylglyoxal were evaluated by GC/MS, after derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride [24]. The approach employed by Tsukushi *et al.* [25], based on ultrafiltration on Centricon membrane (Millipore, Bedford, MA, USA), was used to deproteinize plasma and dialysate samples. The derivatization procedure was exactly the same as that described elsewhere [26]. Glyoxal and methylglyoxal levels were expressed as μg/L.

2.3 Statistical analysis

Values are expressed as means ± SD. Differences between diabetic patients at various observation times (A, B, C) and control subjects (D) were evaluated by one-way ANOVA, followed by the unpaired *t*-test with Bonferroni's correction for multiple comparisons, assuming three comparisons of interest. Pairwise multiple comparisons among diabetic patients at times A, B and C were done by means of ANOVA for repeated measures, followed by the paired *t*-test with Bonferroni's correction.

In the study involving patients with end-stage renal disease, due to the small number of patients, non-parametric tests were used. Pairwise comparison of quantitative variables before and after treatment was done using the Wilcoxon matched-pairs signed-ranks test. The comparison between patients and controls was obtained using the Mann-Whitney test for unpaired data.

Linear regression analysis was used to establish possible relationships between analyzed parameters and calculated by the least-squares method. Regression significance was tested against zero with Student's *t*-test.

For all statistical analyses, a p-value <0.05 was considered significant (two-tailed).

3 Results

3.1 Diabetic patients

At baseline, the mean values of fasting plasma glucose (FPG), HbA1c, glyoxal, methylglyoxal and pentosidine were significantly higher ($p < 0.001$) in diabetic patients than in controls (Table 1).

At the second patient evaluation, 2 months later, the mean values of plasma glucose and HbA1c were significantly lower ($p < 0.001$) than those obtained at baseline. Mean values of glyoxal and methylglyoxal had reduced significantly ($p < 0.05$ and $p < 0.01$, respectively), and pentosidine showed a tendency to reduce (although not significant), as shown in Table 1.

Table 1. Mean values (± SD) of parameters evaluated in normal controls (D) and diabetic patients at baseline (A), and after 2 months (B), and after 10 months (C)

	Fasting plasma Glucose (mg/dL)	HbA1c (%)	Pentosidine (pmol/mL)	Glyoxal (μg/mL)	Methylglyoxal (μg/mL)
A)	311.4 ± 82.8[a, b)]	12.9 ± 2.3[a, b)]	166.5 ± 33.0[b2)]	27.2 ± 8.6[a2)]	29.3 ± 5.5[a1)]
B)	151.2 ± 36.0[c)]	7.6 ± 1.3[c)]	145.6 ± 28.0[c)]	20.6 ± 5.2	26.5 ± 5.9
C)	130.4 ± 35.2	6.6 ± 0.9	98.2 ± 27.0	–	–
D)	89.0 ± 4.8 ***	5.6 ± 0.4 ***	63.0 ± 1.4 ***	12.5 ± 0.5 ***	8.5 ± 0.5 ***

Differences between diabetic patients at various times (A, B, C) *versus* control subjects (D) were evaluated by using one-way ANOVA followed by unpaired *t*-test with Bonferroni correction for multiple comparisons. All the three groups of diabetics resulted significantly different from controls (*** $p < 0.001$). Pairwise multiple comparisons among diabetic patients at various times (A, B, C) were carried out by means of ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction, assuming three comparisons (except for glyoxal and methylglyoxal, where no multiple comparisons were possible).
Significance levels:
a) $p < 0.001$; a1) $p < 0.01$; a2) $p < 0.05$ for A *versus* B
b) $p < 0.001$; b2) $p < 0.01$ for A *versus* C
c) $p < 0.01$ for B *versus* C

On the third evaluation, after 10 months, significantly lower values of plasma glucose ($p < 0.001$), HbA1c ($p < 0.001$) and pentosidine ($p < 0.01$) were found with respect to baseline values, and also in comparison with the observation after 2 months ($p < 0.01$). However, whereas HbA1c levels were nearly normal, pentosidine levels remained high (Table 1). No data were available for glyoxal and methylglyoxal after 10 months.

In diabetic patients, the percentage decrease of FPG was 49.8% at 2 months and 49.7% at 10 months; HbA1c was 40.6% at 2 months and 44.5% at 10 months; pentosidine was 12.2% at 2 months and 28.2% at 10 months; glyoxal and methylglyoxal were 15 and 7.9% at 2 months, respectively.

HbA1c levels showed positive relationships with fasting plasma glucose values at baseline ($r = 0.6$, $p < 0.05$), after 2 months ($r = 0.65$, $p < 0.05$) and 10 months ($r = 0.69$, $p < 0.005$). No relationship was found between glyoxal, methylglyoxal, pentosidine and plasma glucose values at time 0 ($r = 0.18$, p = ns; $r = 0.20$, p = ns; $r = 0.19$, p = ns, respectively), and after 2 months ($r = 0.17$, p = ns; $r = 0.18$, p = ns; $r = 0.16$, p = ns, respectively). Finally, plasma glucose levels were not related to pentosidine values after 10 months ($r = 0.18$; p = ns).

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3.2 Patients with end-stage renal disease

The mean values of pentosidine and free pentosidine are reported in Table 2. In plasma, mean values of pentosidine had decreased after 12 h of peritoneal dwell time ($p < 0.01$). These values were always higher than those obtained from normal controls ($p < 0.001$). Free pentosidine levels had also decreased after 12 h dwell time ($p < 0.01$); no free pentosidine was detected in the plasma of normal controls.

The percentage decrease of free-pentosidine and pentosidine in plasma of patients with end-stage renal disease were 29 and 9%, respectively.

In the case of peritoneal dialysate, no pentosidine or free pentosidine were found at time 0, while after 12 h of peritoneal dwell time a significant level of both metabolites was detected, the mean values being 25.9 ± 15.7 pmol/mL for pentosidine ($p < 0.01$) and 19.8 ± 12.5 pmol/mL for free pentosidine ($p < 0.01$).

The mean values (± SD) of glyoxal and methylglyoxal levels obtained in plasma and dialysate samples, at time 0 and after 12 h of peritoneal dwell time, are presented in Table 2. The mean values of glyoxal in plasma showed a significant decrease after 12 h dwell time ($p < 0.01$). Plasma methylglyoxal was lower after 12 h dwell time, although not significantly. Both values were significantly higher than those obtained in normal controls ($p < 0.001$). The percentage decreases of methylglyoxal and glyoxal in plasma were 12 and 20%, respectively.

An analogous trend was observed in dialysate, which had significant decreases in methylglyoxal ($p < 0.01$) and glyoxal ($p < 0.01$), with percentage decreases of 65 and 49%, respectively.

Table 2. Mean values (±SD) of free pentosidine, pentosidine, methylglyoxal and glyoxal in peritoneal dialysate and plasma of patients with end-stage renal disease at $t = 0$ and after 12 h dwell time

	Peritoneal Dialysate		Plasma	
	$t = 0$	$t = 12$ h dwell time	$t = 0$	$t = 12$ h dwell time
Free pentosidine (pmol/mL)	0	19.8 ± 12.5[a)]	40.2 ± 20.0	28.6 ± 16.3[a)]
Pentosidine (pmol/mL)	0	25.9 ± 15.7[a)]	748.3 ± 407.5	678.8 ± 362.8[a)]
Methylglyoxal (μg/mL)	23.3 ± 4.0	8.2 ± 1.2[a)]	17.5 ± 6.9	15.3 ± 6.1 NS
Glyoxal (μg/mL)	24.3 ± 4.7	12.5 ± 2.6[a)]	22.6 ± 3.0	18.1 ± 3.9[a)]

a) $p < 0.01$, Wilcoxon matched-pairs signed-ranks test, comparison between $t = 0$ and $t = 12$ hours.
NS: not significant

4 Discussion

"Glycoxidation" is the term used for glycation processes involving oxidation. High intracellular glucose concentration is an important trigger for increased glycation, leading to increased formation of methylglyoxal, glyoxal, and 3-deoxyglucosone. These products can glycate proteins and form intracellular and extracellular AGE. Oxidative stress enhances these processes and is, in its turn, enhanced by AGE/RAGE interactions [27].

We verified the possible relationships between some dicarbonyl compounds (glyoxal and methylglyoxal), pentosidine, a well-known AGE, and the common metabolic parameters (plasma glucose and HbA1c) used in diabetic patients. Due to the intensive insulin treatment and strict follow up, type 2 diabetic patients had improved metabolic control after 2 months, as shown by the percentage decrease of fasting plasma glucose (49.8%) and HbA1c (40.6%). Glyoxal, methylglyoxal and pentosidine also decreased, but by less (15, 7.9 and 28.2%, respectively). This behavior may be ascribed to the different formation pathways of these substances. HbA1c represents the amount of ketoamine formed on hemoglobin during the early stage of non-enzymatic glycation reaction. In this stage the reaction is reversible and leads to an unstable aldimin that further rearranges to a stable ketoamine, the Amadori product. *In vivo* the Amadori product reaches equilibrium after approximately 15 to 20 days depending on a series of variables (plasma glucose, protein concentrations, protein half-life and their reactivity). Glyoxal and methylglyoxal are formed in the intermediate stage of the Maillard reaction and their formation requires oxidation conditions. Lastly, pentosidine is a glyco-oxidation product formed in the late stage of this reaction, which is stable and irreversible [1, 3].

It should nevertheless be pointed out that, after 2 months, the metabolic control of the patients in the study was improved but not optimal, as shown by the mean values and SDs of HbA1c. It can thus be hypothesized that a longer period of good metabolic control might be necessary to reduce glyoxal, methylglyoxal, and pentosidine.

We therefore decided to prolong our observation and revaluate the patients after 10 months: on that occasion, we were only able to evaluate pentosidine levels. Plasma glucose levels, and especially HbA1c values, were much reduced and close to normal in the patients, but pentosidine levels, although lower, had not normalized. So, unlike HbA1c, a long period of good metabolic control is unable to normalize the products of the late stage of the Maillard reaction. These data can be explained by the fact that independently of the protein turnover, hyperglycemia induces persisting oxidative stress that is able per se, and independently of the

glucose concentration, to induce and potentiate AGE formation in diabetic patients.

Moreover, no correlations were found at any time between glyoxal, methylglyoxal, pentosidine and plasma glucose, while there was a linear relationship between HbA1c and plasma glucose improving with the better metabolic control.

As regards dicarbonyl compounds and AGE in patients with end-stage renal disease, we observed a pentosidine and free pentosidine reduction in plasma and the appearance of these compounds in the peritoneal dialysis medium after 12 h of dialysis. This finding confirms what has previously been shown on the effectiveness of peritoneal dialysis in clearing these compounds [28].

In contrast with the data of Friedlander *et al.* [28], we found no pentosidine in peritoneal dialysis fluid at time 0. In our study, the sample at time 0 was collected from bags before infusion into the peritoneal cavity. In the above-mentioned study, the pentosidine sample at time 0 was obtained immediately after the exchange with Dianeal. Finding no free pentosidine in healthy controls, due to its very low concentration, agrees with the data of Myata *et al.* [5] and disagrees with Agalou *et al.* [29]. This may be due to the different methods used [16].

Surprisingly, different behavior is observed for glyoxal and methylglyoxal: these compounds had reduced in both plasma and peritoneal medium after 12 h of dialysis, even if the reduction for methylglyoxal in plasma was not statistically significant. The presence of these dicarbonyl compounds at time 0 in the dialysis medium confirms previous reports in the literature [18, 19] showing that they can be formed during heat sterilization of the dialysis solutions. Our results demonstrating a clear reduction of glyoxal and methylglyoxal in dialysate after 12 h of dialysis agree in part with those of Ueda *et al.* [30] and Miyata *et al.* [31] showing a reduced concentration of these compounds after 12 h dwell time. Furthermore, the reduction of glyoxal and methylglyoxal in plasma and dialysate after 12 h dwell time, and the parallel increase of pentosidine and free pentosidine in the dialysate, seems to indicate that glyoxal and methylglyoxal are a source of formation of pentosidine in the peritoneal cavity, thus leading to impairment of peritoneal membrane functionality.

The results of our study suggest that in these conditions, the measurement of glyoxal, methylglyoxal and pentosidine may be important for evaluating the real oxidative stress experienced by the patients. Equally, the measurement of pentosidine plasma levels may be useful in evaluating the "very" long term metabolic control of diabetic patients.

Our data also support the hypothesis of a transformation of dicarbonyl compounds into AGE at peritoneal membrane level, so determining alterations of structure of the peritoneal matrix protein [31, 32] and providing a reason for the gradual loss of peritoneal membrane function often found in patients undergoing long-term peritoneal dialysis [31, 32].

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