Plasma levels of advanced glycation end products during haemodialysis, haemodiafiltration and haemofiltration: potential importance of dialysate quality*

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Abstract

Background. Advanced glycation end products (AGEs) accumulate in patients with end-stage renal disease (ESRD). The aim of this study was to investigate the potential influence of different modalities of renal replacement therapies on plasma AGE levels. Methods. The removal of AGEs by high-flux haemodialysis (HD) using standard and ultrapure dialysis fluid (SDF and UDF), by haemodiafiltration (HDF) and by haemofiltration (HF) was studied by fluorescence spectroscopy and by a carboxymethyllysine (CML)-specific ELISA. In addition, molecular weight distribution of fluorescent AGE products in serum of several patients was analysed by gel filtration.

Results. The highest AGE-typical fluorescence was found in the serum of patients on HD using SDF (114 667 ± 18 967 arbitrary units (AU)), followed by patients on HDF (86 912 ± 24 411 AU, \( P < 0.005 \)), by patients on HD using UDF (74 953 ± 21 152 AU, \( P < 0.0001 \)) and by patients on HF (74 039 ± 17 027 AU, \( P < 0.0001 \)). Similar results were found for serum CML levels with the highest values in HD patients on SDF (1609 ± 504 ng/ml), followed by patients on HDF (1354 ± 614 ng/ml, \( P < 0.001 \)), then by HD patients on UDF (1310 ± 403 ng/ml, \( P < 0.001 \)) and by patients on HF (1132 ± 338 ng/ml, \( P < 0.001 \)). The removal rate of AGEs, as evaluated by the determination of the pre-/post-dialysis AGE differences, was comparable across all groups.

Conclusion. These findings suggest that factors other than removal are responsible for the lower pre-dialysis AGE levels found in patients on convective dialysis as well as on HD with UDF. A role of water quality is assumed. This is corroborated by the finding that the high molecular weight AGE-fraction is preferentially lowered in comparison with patients on HD with SDF, as analysed by gel filtration chromatography. These findings could be best explained by a less severe oxidative stress (i.e. resulting in decreased AGE generation) with HF and HDF, as well as with ultrapure HD.

Keywords: advanced glycation end products; end-stage renal disease; haemodiafiltration; haemodialysis; haemofiltration

Introduction

Advanced glycation end products (AGEs) are thought to be involved in many complications of diabetes as well as those of end-stage renal failure [1–3]. These compounds are formed by a non-enzymatic reaction between reducing sugars or other reactive glycation products and primary amino groups of proteins. They form a structurally diverse class of heterocyclic molecules which exhibit fluorescence, cross-linking properties and have a yellow–brown colour [4].

Patients with end-stage renal disease (ESRD) have very high AGE levels [5–7]. The main factors involved are increased AGE formation by enhanced carbonyl and oxidative stress, and impaired AGE removal by the damaged kidney [8]. In ESRD, dialysis therapy may contribute to the removal of AGEs, particularly AGE peptides [6]. It is therefore of interest to investigate the influence of different modalities of renal replacement therapies. In this study, the removal of AGEs by high-flux haemodialysis (HD) using standard
dialysate fluid (SDF) and ultrapure dialysate fluid (UDF).
by haemodiafiltration (HDF) and by haemofiltration (HF)
was examined by fluorescence spectroscopy and
by an enzyme-linked immunosorbent assay (ELISA);
in addition, the molecular weight distribution of fluore-
cent AGE-products in the serum of several patients
was analysed by gel filtration chromatography.

Patients and methods

This cross-sectional study was performed in 71 stable dialysis
patients with long-term ESRD. Four patient groups were
formed: patients on haemodialysis with SDF \(n = 18\), HD
patients with UDF \(n = 17\), patients on HDF \(n = 18\) and
patients on HF \(n = 18\). Main patient characteristics are
given in Table 1. Residual function was \(<100\) ml urine/24 h.
GFR was therefore not determined. Eleven healthy subjects
served as controls.

Dialysis treatment modalities

In all patients, three dialysis sessions per week for \( \sim 4.5 \) h
took place. The adequacy of dialysis was measured by urea
reduction ratio (>65%) and \( \text{Kt/V} (>1.2) \) values. No dialyser
was re-used.

Maintenance HD was performed as follows: blood flow
250–300 ml/min, dialysate flow rate 500 ml/min. Dialysers
were high-flux polysulfone dialysers (F 60S or HF 80S;
Fresenius Medical Care, Bad Homburg, Germany). Standard
dialysis fluid was prepared from purified water obtained from
reverse osmosis and mixed with the acid electrolyte con-
centrate (SKF213 with a potassium content of 2.0 mVal/l or
SKF313 with a potassium content of 3.0 mVal/l; Fresenius
Medical Care) and the liquid bicarbonate solution obtained
from NaCO3 powder (Bilog; Fresenius Medical Care).

Microbial contamination of SDF was determined monthly
in the dialysate at temperatures of 22°C and 36°C for a pro-
longed incubation time of 7 days. All bacterial counts were in
the range of \(0–70\) colony-forming units (CFU)/ml and thus
met the standards of the Association for the Advancement
of Medical Instrumentation (\(<200\) CFU/ml in water,
2000 CFU/ml in dialysate). The endotoxin level in the
dialysate was measured by the limulus amoebocyte lysate
(LAL) assay. Our data was markedly less than the limit of
measurability (\(0.03\) EU/ml) [9], which is in line with our data.

UDF was achieved by placing an additional ultrafilter
(polysulfone, 0.1 \(\mu\)m) close to the dialyser. UDF is char-
acterized by a bacterial contamination of \(<0.1\) CFU/ml and
unmeasurable endotoxin levels (\(<0.03\) EU/ml) [9], which is
in line with our data.

Table 1. Patient groups

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Duration (years)</th>
<th>Duration (non-diabetics)</th>
<th>Duration (diabetics)</th>
<th>Albumin (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD SDF</td>
<td>53±11</td>
<td>8±4</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>HD UDF</td>
<td>56±12</td>
<td>8±3</td>
<td>17</td>
<td>–</td>
</tr>
<tr>
<td>HDF</td>
<td>61±17</td>
<td>7±5</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>HF</td>
<td>71±9</td>
<td>7±3</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>53±9</td>
<td>–</td>
<td>11</td>
<td>–</td>
</tr>
</tbody>
</table>

HD, haemodialysis; HDF, haemodiafiltration; HF, haemofiltration;
SDF, standard dialysate fluid; UDF, ultrapure dialysate fluid.

Post-dilution haemodiafiltration (HDF) was performed
using AK 200 machines (Gambro, Hechingen) as well as a
specially equipped Fresenius 4008 machine. Haemodia-
filters were HF60 and HF80, depending on the body surface.
Ultrapure water was used both for the dialysate and for
online production of the highly purified substitution fluid.
Blood flow averaged 300–350 ml/min, total dialysate flow
650 ml/min, dialysate flow 500–550 ml/min, infusate flow
\(~100\) ml/min. Total filtrate volume was aimed at one-third
of the body weight, corresponding to \(20–25\) l per session.

Online HDF (post-dilutional method) was performed by
use of AK 200 ULTRA machines (Gambro), as well as
Online PLUS 4008 machines from Fresenius. Haemodia-
filters were HF60 and HF80, depending on the body surface.
Ultrapure water was used both for the dialysate and for
online production of the highly purified substitution fluid.
Blood flow averaged 300–350 ml/min, total dialysate flow
650 ml/min, dialysate flow 500–550 ml/min, infusate flow
\(~100\) ml/min. Total filtrate volume was aimed at one-third
of the body weight, corresponding to \(23–27\) l per session.

In a similar manner, post-dilutional haemofiltration (HF)
was performed using AK 200 ULTRA machines, and HF60
and HF80 haemofilters. Total fluid exchange was identical to
that of HDF.

Blood sampling

After receiving informed consent, blood samples of the
patients were collected at midweek and endweek sessions
before (prior to heparin administration) and after the therapy
session (<5 min). Samples were collected into Sarstedt
monovettes, centrifuged for 10 min and the serum stored at
\(-20\)°C before determination.

Fluorescence spectroscopy

The fluorescence measurement of the 50-fold diluted serum
samples (corrected for background) was performed in
triplicate on a FluoroMax spectrometer (Spex Instruments,
Edison, NJ, USA) at a wavelength of 440 nm (excitation
370 nm) as described previously [10]. Haemolytic sera were
excluded. No significant shift of the fluorescence maximum
was observed after dialysis.

Measuring the content of N-(carboxymethyl)lysine
(CML) by competitive ELISA

Serum CML concentrations were measured in triplicate by a
competitive ELISA developed by ROCHE Diagnostics
(Penzberg, Germany) using the anti-CML monoclonal anti-ody 4G9 (Alteon Inc., New York, NY, USA) as described
previously [11]. Briefly, Proteinase-K-digested serum (to
make hidden epitopes accessible) was incubated on AGE–
BSA-coated, BSA-blocked microtitre plates in the presence
of peroxidase-conjugated anti-CML monoclonal antibody
for 1 h. After three washing steps, colour reaction was
induced with 2,2’-azino-di-3-ethylbenzthiazoline-sulfonic
acid (Roche Diagnostics) and 0.01% \(\text{H}_2\text{O}_2\) in 0.01%
glycine/citrate buffer and absorbance was read in a microtitre
ELISA plate reader (Multiskan Ascent; Labsystems, Helsinki,
Finland) at 405 nm. All steps were carried out at room
temperature. N-(carboxymethyl)-l-arginine/caproic acid (Alteon
Inc.) served as standard.
**Gel filtration chromatography**

Molecular weight distribution of serum AGEs was determined by gel filtration chromatography. The separation was performed by fast protein liquid chromatography (Biologic System BioRad; BioRad, Hercules, CA, USA) on a Superdex 75 HR 10/30 column (Pharmacia, Freiburg, Germany) equilibrated with PBS, the flow rate being 1 ml/min. Fluorescence was measured at an emission wavelength of 440 nm (excitation 370 nm) with a Merck-Hitachi F-1080 fluorospectrometer (Hitachi, Tokyo, Japan).

**Statistics**

Results are expressed as mean ± SD. Comparison between different groups was performed by one-way analysis of variance (ANOVA) using the SPSS program package (SPSS Inc., Chicago, IL, USA). Statistical significance was defined at *P* < 0.05.

**Results**

**Fluorescence measurements**

When AGE levels in serum of controls (*n* = 11) were compared with serum AGE levels of dialysis patients, a significant difference was found (*P* < 0.0001) between control and all treatment modalities (Figure 1). Figure 1 shows that there are also differences in AGE levels in the serum of dialysis patients on different therapy forms. The highest fluorescence at predialysis was found in serum of HD patients using SDF, followed by patients on HDF and HF (Table 2). Fluorescence levels of patients on HD using UDF were found in the same range as for patients on HF (Table 2). The level of AGE-typical fluorescence of the HDF patients and HF patients was ~24% (*P* < 0.005) and ~35% (*P* < 0.0001) lower, respectively, than that of patients on HD with SDF. The use of UDF lowered serum fluorescence by 35% (*P* < 0.005). The differences were not significant between HDF and HD with UDF, between HF and HD with UDF, or between HDF and HF. In the course of a dialysis session, the serum levels of AGE-typical fluorescence declined by 24% (HD with UDF, *P* < 0.001), 28% (HD with SDF, *P* < 0.001), 35% (HF, *P* < 0.001) and 23% (HDF, *P* < 0.001) (corrected for haemo-concentration according to Bergstrom and Wehle [12]) (Table 3). The differences in the reduction rate of the AGE-typical fluorescence were not significant. The post-dialysis AGE-typical fluorescence levels of HF and HDF were significantly lower than those of HD patients on SDF (*P* < 0.001).

**AGE levels in serum measured by competitive ELISA**

In accordance with the fluorescence measurements, the ELISA of CML showed a significant difference between dialysis patients and controls (*P* < 0.0001) for all three renal replacement therapies (Figure 1). Also, significantly lower CML levels were observed for patients on HDF and HF (*P* < 0.001) when compared with HD patients with SDF. CML levels of patients on HD with UDF were found to be intermediate, being significantly lower than levels of patients on HD with SDF, but having slightly higher levels of CML compared with HDF (*P* > 0.1, not significant) (Table 2).
The differences between HD with UDF and HF as well as HDF vs HF were not significant. Serum CML values of HF patients were slightly higher (1354 ± 614 ng\text{ml}) than those of HDF patients (1132 ± 338 ng\text{ml}) (P = 0.1). The average reduction of CML within one dialysis session was 28% (P < 0.001) for HD with SDF, 28% (P < 0.001) for HD with UDF, 32% (P < 0.001) for HDF and 31% (P < 0.001) for HF. There was no significant difference between the different dialysis modalities with respect to CML reduction (Table 3).

Gel filtration chromatography

Molecular weight distribution of serum AGEs was determined by gel filtration chromatography on a Superdex column (separation range 3–70 kDa). Serum samples of five patients of each subgroup (SDF HD, UDF HD, HDF, HF) were analysed and compared with controls (Figure 2). While differences in serum fluorescence between HD patients and controls are due to considerably higher levels of both the high- (MW >12 kDa) and low-molecular weight (MW <12 kDa) fluorescent AGEs, the dissimilarity in overall fluorescent AGE concentrations of the patients on different dialysis modalities appears to be mainly due to a lower AGE modification of proteins, in particular albumin, as judged from the quantification of the gel filtration chromatograms (Table 4).

Discussion

In various studies it has been demonstrated that removal of AGEs by dialysis is inadequate, mostly due to the fact that the majority of AGEs are protein-bound. Thus, only small percentages of total pentosidine can be removed by dialysis [13]. In the case of CML and fluorescent AGEs, higher reduction rates in the range of 20% were reported [14,15]. Furthermore, it could be shown that the chemical nature of the dialysis membranes may have a higher impact on AGE levels than the pore size [16], giving rise to the suspicion that AGE generation by polymers may override their removal capacity. The original intention of this study...
was to show whether——apart from pore size and chemical nature of membranes——dialysis modalities such as haemofiltration and haemodiafiltration can influence serum AGE levels, since the latter treatment modalities have been reported to remove peptides more efficiently than high-flux dialysis [17]. At first glance, in agreement with these findings, we found that the pre-dialysis AGE levels of patients on haemodiafiltration and haemofiltration are significantly lower than those of patients on high-flux haemodialysis using standard dialysis fluid. An effect of the chemical nature of the membranes is unlikely, since only polysulfone membranes were used. However, no significant difference in AGE removal rate could be found for haemodialysis and haemofiltration/haemodiafiltration. Therefore, the lower pre-dialysis AGE levels of patients on HDF and HF cannot be easily explained by more efficient removal during therapy session, although long-term effects of small, immeasurable differences cannot be excluded. Another possible source of raised AGE serum levels can be increased formation. Miyata and colleagues (2000) have found that there is considerably enhanced carbonyl and oxidative stress in dialysis patients, which might contribute substantially to the heightened levels of AGES [8]. Hence, less oxidative stress may be present in patients on haemodiafiltration and haemofiltration compared with high-flux haemodialysis (using SDF), although the same dialysis membranes are used in all modalities. This is corroborated by the fact that the concentration of high molecular weight AGES—in particular albumin-bound AGES—is lowered in HF and HDF. This cannot be achieved by removal of AGES but may be better explained by a more efficient removal of AGE precursors such as dicarboxyls, oxidants and free radicals, leading to reduced AGE generation.

An important factor to be discussed as a possible promotor of oxidative stress is the quality of dialysis fluid. Water containing pyrogens may stimulate pro-inflammatory cytokines and acute phase proteins [9]. Even a low-grade bacterial contamination of the dialysate (<200 CFU/ml) may exert toxic effects with increased release of radicals and cytokine formation from the mononuclear cells (the so-called cytokine-inducing substances) [9]. Naturally, for haemofiltration and haemodiafiltration, only sterile and double-filtered pyrogen-free substitution fluid is used. Apart from convective transport, another important difference between haemodiafiltration and haemodialysis with SDF is the use of ultrapure dialysis fluid as well as highly purified sterile infusions. To test the role of water quality, AGE levels of haemodialysis patients using standard or ultrapure dialysis fluids were compared. The results suggest an influence of water quality on AGE levels, although the bacterial counts in the HD patients on standard dialysis met with criteria of the Association for the Advancement of Medical Instrumentation (Figures 1 and 2). Also, the LAL test was within normal limits.

According to our studies, we assume that in the case of AGE-typical fluorescence, water purity of the dialysate and/or of the infusion fluid may account at least partially for the observed reduction in pre-dialysis AGE levels of patients on haemofiltration or haemodiafiltration. Our results indicate that, besides many other potential factors (e.g. residual renal function, membrane properties, oxygen radical formation and nutritional factors), water quality appears to have an impact on serum AGE levels of dialysis patients as well.

References