ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Mar; 21 (3): 276 - 280
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Clearance of increased serum advanced glycosylation end products in patients with end stage renal disease by hemodialysis[1]

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KEY WORDS advanced glycosylation end-products; kidney diseases; flow injection analysis; enzyme-linked immunosorbent assay; fluorescence spectrometry; hemodialysis

AIM: To study the effect of removal of hemodialysis using acetate membrane on serum advanced glycosylation end products (AGEP) in 36 patients (59.1 ± 1.6) with end stage renal disease (ESRD). METHODS: Serum AGEP levels were determined with quantitative fluorescence spectrometry, flow injection analysis (FIA), and competitive enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody directed against AGEP. RESULTS: The serum AGEP levels in patients with ESRD quantified by fluorescence spectrometry, FIA, and ELISA were higher than those in controls [(25 ± 5) *vs* (7.5 ± 1.5), (6.1 ± 1.8) *vs* (1.4 ± 0.5), and (37 ± 20) *vs* (9 ± 10) kU/L, respectively] and markedly reduced to [(22 ± 6), (4.2 ± 1.4), and (19 ± 14) kU/L, respectively] after hemodialysis. CONCLUSION: Increased serum AGEP levels in the circulation of patients with ESRD were reduced effectively by hemodialysis.

INTRODUCTION

Advanced glycosylation end products (AGEP) are generated by the non-enzymatic reaction of reducing sugars with the free amino group of proteins or amino acids[1]. They exhibit characteristics browning, fluorescence, intra- and intermolecular covalent crosslinks. AGEP in the circulation may be a heterogeneous unrecognized class of potentially toxic substances. A large number of studies suggest that the various chronic complications of diabetes and end stage renal disease (ESRD) result from the accumulation of tissue macromolecules that have been progressively modified by AGEP[2,3]. The elevation of AGEP level in sera of patients with diabetes and ESRD is well confirmed, serum AGEP levels in patients with ESRD is found to be higher than in patients with diabetes[4,5]. Removal of AGEP from serum delays the onset and slows the progression of complications in patients with diabetes and ESRD. There is a controversy whether hemodialysis can reduce the increased serum AGEP level[6,7]. In the present study, we detected the serum AGEP level in patients with ESRD before and after hemodialysis by quantitative fluorescence spectrometry, flow injection analysis (FIA), and enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Reagents Bovine serum albumin (BSA), human serum albumin (HSA), gelatin, *O*-phenylenediamine (OPD) and proteinase K were purchased from Sigma. Biotinylated anti-rabbit IgG antibody and horseradish peroxidase (HRP) linked streptavidin were obtained from Vector. Other chemicals were of AR.

Serum samples Serum samples were obtained from 54 control subjects [age: (59.1 ± 1.6)] a and 36 patients with ESRD pre- and post-hemodialysis [age: (65 ± 4) a, (6.9 ± 2.0) a of dialysis periods]. The intervals between dialysis sessions were 48 - 72 h. The serum samples of post-hemodialysis were collected within 30 min after hemodialysis. Serum samples were stored at - 20 ℃ till analysed.

Clinical data The primary causes of ESRD in this study group were chronic glomerulonephritis, hypertension, chronic interstitial nephritis, polycystic kidney disease and unknown causes. Patients with diabetic nephropathy were excluded. All the patients accepted the conventional bicarbonate dialysis with blood flow 250 - 300 mL/min, dialysate flow 500 mL/min. Each dialysis session took 4.5 - 5 h, using acetate membrane (1.3 m^2). The

[1] Project supported by the National Natural Science Foundation of China, № 39470314.
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Received 1999-03-26 Accepted 1999-09-30

average serum creatinine (pre-HD) was (459 ± 95) μmol/L.

Preparation of AGEP-BSA and AGEP-HSA BSA or HSA 50 g/L were incubated with *D*-glucose 0.5 mmol/L in the phosphate buffer saline (PBS, pH 7.4) 0.2 mmol/L at 37 ℃ for 90 d. The control samples of BSA or HSA were also incubated under same conditions but without glucose. After incubation, dialysis against PBS was carried out to remove unbound glucose. Fluorescence spectra were recorded using a 650-60 fluorospectrometer (Hitachi, Japan). AGEP-BSA and AGEP-HSA were purified by Sephadex G-200. The method of Bradford was used for quantification of proteins.

Quantitative fluorescence spectrometry Serum sample 50 μL was diluted with PBS upto 5.0 mL. After the samples were filtrated through 0.22 μm filters, the fluorescence intensity was measured with a 650 – 60 fluorospectrometer at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. Various dilutions of purified AGEP-BSA were used as calibrator and the sample AGEP levels were calculated according to the standard curve. The AGEP value was defined as U/mL, 1.0 U/mL equals to AGEP-BSA 1.0 mg/L.

Competitive ELISA The polyclonal rabbit antibody was raised against purified AGEP-BSA as previously reported[8]. The competitive ELISA was performed in triplicate with some modification[8]. Briefly, 96-well microtitre plates (Nunc, Denmark) were coated with AGEP-HSA (100 μL/well, 5 mg/L) overnight and blocked with 200 μL/well of 0.5 % gelatin for 1 h. After washing six times with PBS containing 0.05 % Tween-20, each well was added 50 μL of serum sample (diluted 1:4) to be tested or calibrator (purified AGEP-BSA diluted as 0.625, 2.5, 10, 50, 100 mg/L) and 50 μL anti-AGEP antibody (1:2000). After incubation for 1 h and washing, 100 μL of biotinylated anti-rabbit IgG antibody (1:4000) was added. Following incubation for 1 h and washing again, the plates were reacted with horseradish peroxidase (HRP) linked streptavidin. Color was developed with OPD and determined at absorbance 490 nm. The AGEP values of sample could be calculated automatically by microplate reader (Bio-rad 550, USA) according to its calibration curve. One AGEP unit was defined as the amount of antibody reactive material that was equivalent to AGEP-BSA standard 1.0 mg/L.

Flow injection analysis[9] The 20 μL of serum samples were mixed with trichloroacetic acid (0.15 mol/L) 480 μL and chloroform 100 μL in microcentrifuge tubes. The tubes were centrifuged (10 min, 13 000 × *g*) to complete the precipitation of proteins and were shaken vigorously to extract lipids into the organic phase. The 20 μL of the aqueous layer was injected to sample injector (loop 20 μL) of high performance liquid chromatography. Water flow rate was at 0.5 mL/min and spectrofluorometric detector was set with emission wavelength at 440 nm and excitation wavelength at 370 nm for detection of AGEP-peptide. The samples were analyzed in triplicate and peak height mode was used for signal measurement. Standard AGEP-peptides (obtained by hydrolysis of AGEP-BSA with proteinase K) were diluted as 0.1, 0.5, 1, 5, 10, 50, 100 mg/L and used for preparing calibration curve and calculating sample AGEP-peptide as described above. The value was also defined as kU/L, 1.0 kU/L equals to standard AGEP-peptide obtained from hydrolysis of AGEP-BSA 1.0 mg/L.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared by paired or unpaired *t*-test and ANOVA.

RESULTS

AGEP determined by ELISA (E-AGEP) The serum E-AGEP levels in patients with ESRD were markedly increased as compared with those in controls [(37 ± 20) *vs* (9 ± 10) kU/L, $P < 0.01$]; The serum E-AGEP levels in patients with ESRD were markedly decreased by 48.5 % after hemodialysis (19 ± 14 kU/L) (Tab 1).

Tab 1. AGEP level in patients with ESRD pre- and post-hemodialysis determined by three independent assays. [a]$P > 0.05$, [b]$P < 0.05$, [c]$P < 0.01$ *vs* controls. [d]$P > 0.05$, [e]$P < 0.05$, [f]$P < 0.01$ *vs* pre-hemodialysis.

	n	F-AGE	E-AGE	AGE-peptide
Control	54	7.5 ± 1.5	9 ± 10	1.4 ± 0.5
ESRD	36	25 ± 5[c]	37 ± 20[c]	6.1 ± 1.8[c]
ESRD + Hemodialysis	36	22 ± 6[cf]	19 ± 14[cf]	4.2 ± 1.4[cf]

AGEP determined by fluorescence spectrometry (F-AGEP) The serum F-AGEP levels in patients with ESRD were markedly elevated compared with those in controls [(25 ± 5) *vs* (7.5 ± 1.5) kU/L, $P < 0.01$]; The serum E-AGEP levels in patients with ESRD significantly decreased by 12.1 % after hemodialysis [(22 ± 6) kU/L] (Tab 1).

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Mar; 21 (3)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

AGEP determined by FIA (AGE-peptide) The levels of serum AGE-peptide in patients with ESRD were markedly higher than those in control subjects [(6.1 ± 1.8) *vs* (1.4 ± 0.5) kU/L, $P < 0.01$]. The levels of serum AGEP-peptide in patients with ESRD were decreased by 30.9 % after hemodialysis [(4.2 ± 1.4) kU/L] (Tab 1). Almost all patients showed a markedly decreased AGEP-peptide after hemodialysis (Fig 1).

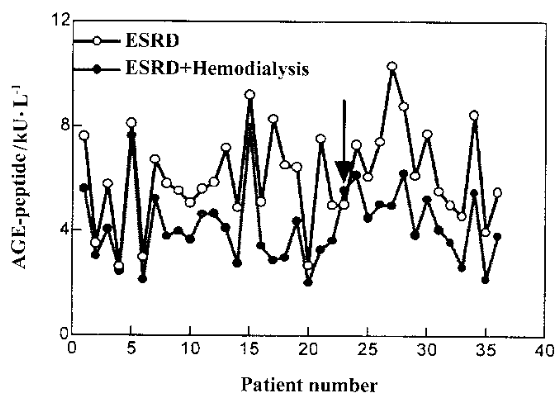

Fig 1. Pre- and post-hemodialysis levels of AGE-peptide in individual patients with ESRD.

Comparison of three analytical approaches The AGE-peptide determined by FIA showed the most excellent precision with lowest inter-assay coefficient of variation (within-day 1.21 %, between-day 6.35 %) and best recovery rate for assay (94.88 % – 101.89 %). Also, it was less time and money consuming. The AGEP level determined by ELISA (E-AGE) showed the highest inter-assay coefficient of variation (within-day 9.51 %, between-day 15.18 %), worst recovery rate (90.92% – 121.43 %) for three analytical approaches. The precision and accuracy of F-AGE (within-day 3.14 %, between-day 7.73 %, recovery rate 95.8 % – 115.13 %) was superior to those of E-AGE (Tab 2).

DISCUSSION

Determination of circulating AGEP is of great value to assess impending cardiovascular risks, to monitor the efficacy of AGEP removal methods, and to test novel pharmacological approaches for inhibition of AGEP formation. However, application of AGEP measurements in clinical practice is still limited by lack of specific, simple, and rapid analytical procedures. Because AGEP produced *in vivo* and prepared *in vitro* by incubation of diverse proteins with diverse reducing sugars contain a common immunological epitope[5,8], it is reasonable and possible to measure the total AGEP in the circulation by this antibody. F-AGE determined by fluorescence spectrometry only represents the AGEP that can produce fluorescence, not including immidazolone, CML, pyrroline[10].

The AGE-peptide just represented the low

Tab 2. Comparison of three analytical approaches for AGEP assay.

	F-AGE	E-AGE	AGE-peptide
Coefficient of variance			
Within-day assay	3.14 %	9.51 %	1.21 %
Between-day assay	7.73 %	15.18 %	6.35 %
Recovery	95.8 % – 115.13 %	90.92 % – 121.43 %	94.88 % – 101.89 %
Sample size	50 μL	50 μL	20 μL
Analysis time	< 10 min	> 6 h	< 20 min
Expenditure (R.M.B)	< 1 yuan	> 5 yuan	< 1 yuan
ESRD/Controls	3.28	4.28	4.4
The number of overlap between ESRD and Controls	0	15	1
The number of decrease case in ESRD post-hemodialysis	24	32	35

molecular weight of AGEP. It is respected that AGE-peptide is a novel and specific marker for monitoring the removal efficacy of AGEP by some procedures.

Each assay for AGEP level showed significant differences between the controls and patients with ESRD or between the pre- and post-hemodialysis. Especially, the AGE-peptide level of all patients with ESRD was significantly decreased after hemodialysis. These results suggest that the clearance of AGEP was mainly mediated by kidney and was markedly impaired in the patients with ESRD, which might play an important role in the development of chronic complications of ESRD. The serum AGEP level determined by three independent assays in patients with ESRD were significantly decreased after hemodialysis, it was valuable to find out that the different parameters of AGEP level for each patient showed different removal effect and the decreased extent of each patient was not similar. These results suggest that the difference for reduced AGEP level could be explained by the fact that the three AGEP parameters reflect heterogeneous classes of AGEP. Although AGEP level could not return to the normal level after hemodialysis, it can be regarded that hemodialysis is a useful and practical therapy for clearing the serum AGEP in patients with ESRD[7]. It is necessary to further investigate the factors that influence the removal efficacy of AGEP or specific portions of AGEP including different modalities of dialysis, the dialysis session, the primary causes of ESRD, the remaining renal function, the age of patients, and so on. It was reported that high flux hemodialysis was more effective (35 % – 40 % AGEP clearance)[11], however, the clinical application of this technology is still limited. Renal transplantation is the best therapeutic modality that can normalize the serum and tissue levels of AGEP[12,13]. Using a lysozyme-linked dialyzer as an adjunctive therapy might be an effective way to eliminate toxic AGEP from the sera of patients with ESRD[14].

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Mar；21（3）
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

血液透析清除终末期肾病患者增高的血清高级糖基化终产物[1]

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关键词 高级糖基化终产物；肾疾病；流动注射分析；酶联免疫吸附测定；荧光光谱法；血液透析

目的：研究血液透析对 36 例终末期肾病患者血中高级糖基化终产物的清除效果. 方法：分别用荧光分光光谱、流动注射分析法和酶联免疫吸附法测定终末期肾病患者血中高级糖基化终产物的水平及比较血液透析前后的变化. 结果：荧光分光光谱、流动注射法和酶联免疫吸附法测定的终末期肾病患者血中高级糖基化终产物的水平显著高于对照组，分别为(25 ± 5) *vs* (7.5 ± 1.5)，(6.1 ± 1.8) *vs* (1.4 ± 0.5)和(37 ± 20) *vs* (9 ± 10) kU/L，透析后上述水平分别降到(22 ± 6)，(4.2 ± 1.4)和(19 ± 14) kU/L. 结论：血液透析能有效地清除终末期肾病患者血中增加的高级糖基化终产物.

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