Original Artcle

Elimination of advanced glycation end products by double filtration plasmapheresis

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Abstract

Objective: To examine the elimination of advanced glycation end products (AGEs) from serum by double filtration plasmapheresis (DFPP) as a potential therapeutic method for reducing glycative stress.

Methods: Subjects consisted of 48 consecutive patients (39 men, 9 women; mean age, 55.3 ± 11.1 years old) with hyper-low-density-lipoprotein (LDL)-cholesterolemia (n = 29) or borderline hyper-LDL-cholesterolemia (n = 12) who received DFPP in our clinic between September 2011 and September 2012. For DFPP, blood was drained from the median cubital vein using heparin as an anticoagulant and was then separated into blood cell and plasma components using a Plasmaflo OP-05W membrane-type plasma separator (Asahi Kasei Medical, Tokyo, Japan). Pathogenic substances were removed from the plasma using a plasma component separator with a blood flow rate of 30 to 60 mL/min, plasma flow rate of 10 to 18 mL/min, and the target plasma treatment volume was estimated to be between 1,500 and 2,000 mL. Pentosidine levels in the serum (n = 16) and waste fluid samples (n = 53) were measured by high-performance liquid chromatography, and N^{ε}- carboxymethyllysine (CML) and malondialdehyde-modified-LDL (MDA-LDL) were quantified by enzyme-linked immunosorbent assay. This research was conducted with the approval of our institutional ethical committee, and informed consent was obtained from all subjects.

Results: The elimination ratios of pentosidine, CML, and MDA-LDL by DFPP from the serum were 14.6% ± 19.3% (n = 16), 35.3% ± 30.0% (n = 15), and 35.8% ± 26.1% (n = 16), respectively. Waste DFPP fluids, which were diluted with 10 mL physiological saline (average total volume: 100 mL), contained 60.7 ± 22.2 pmol/mL of pentosidine (n = 42), corresponding to a total of approximately 6,000 pmol. The waste fluids were also estimated to contain 370 μg CML (3.7 ± 1.7 μg /mL; n = 15) and 44 U of MDA-LDL (n = 53).

Conclusions: The elimination efficiency of serum AGEs by DFPP was similar to that of LDL-C, suggesting that DFPP may have glycative stress-reducing effects. As the removal efficiency of pentosidine was less than that of CML, improvement of DFPP filtration membranes for the increased removal of low-molecular-weight AGEs, such as the combined use of a small-pore-size filter membrane or specific adsorbents, may be necessary to further enhance the reduction of glycative stress by this method.

Introduction

Elevated levels of reducing sugars, aldehydes, and keto acids in the body are results in high glycative stress [1]. Glycative stress leads to the non-enzymatic and irreversible structural and functional modification of proteins, lipids, DNA bases, and amyloids, resulting in the formation of various substances, including advanced glycation end products (AGEs) [1,2] which we have termed glycative stress-induced products (GSIPs). The accumulation of GSIPs in cells and tissues generally increases with age and is associated with various types of damage, such as the formation of cataracts and diabetic nephropathy. The conformational modification of proteins can disrupt their function and cause cellular damage. More than 20 types of receptors are reported to bind GSIPs as activating ligands [2], leading to the downstream activation of transcription factors, such as NF-κB, and the production of inflammatory cytokines. Glycative stress may therefore exacerbate aging processes and have a role in lifestyle-related diseases and diabetic complications.

To reduce glycative stress, approaches to decrease the levels of the causative agents that lead to GSIP accumulation are needed and include therapeutic and pharmaceutical

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interventions, and lifestyle improvements. A number of therapeutic approaches to lower glycative stress are being actively researched and include the inhibition of GSIP formation, promoting the breakdown of GSIPs, and eliminating or antagonizing GSIP receptors. We previously reported that double-filtration plasmapheresis (DFPP) removed pentosidine, a type of AGE, as well as low-density lipoprotein cholesterol (LDL-C), from the serum of patients with hyper LDL-cholesterolemia [3,4]. Although this finding suggests that DFPP reduces AGE levels in serum, the efficacy of this method for the removal of other AGEs remains unclear.

In the present study, we measured the concentration of AGEs in DFPP waste fluids from patients with elevated levels of LDL cholesterol to determine the elimination efficiency of AGEs during DFPP.

Materials and methods

Subjects

The study subjects consisted of 48 patients (39 men, 9 women; mean age, 55.3 ± 11.1 years old; age range, 28 to 71 years old), 29 (22 men and 7 women) with hyper-LDL-cholesterolemia and 12 (9 men and 3 women) with borderline hyper-LDL-cholesterolemia who received DFPP at our clinic, "Center for Advanced Medical Science and Technology, Midtown Clinic Group" (Minato-ku, Tokyo, Japan), between September 2011 and May 2012. A total of 68 samples of waste fluids from the DFPP process were obtained from among the subjects and included samples from subjects who received DFPP on multiple occasions. Serum samples were collected from these subjects before and after DFPP. Prior to enrollment in the study, written consent was obtained from each subject.

DFPP

DFPP was performed as described previously [3]. Briefly, blood was drained from the body via the median cubital vein and was then separated into blood cell and plasma components using a Plasmaflo OP-05W membrane-type plasma separator (Asahi Kasei Medical, Chiyoda-ku, Tokyo, Japan). Pathogenic substances were removed from the isolated plasma using a Cascadeflo EC-50W plasma component separator (Asahi Kasei Medical), and the plasma and blood cells were then returned to the subject. An ACH Σ blood purification device (Asahi Kasei Medical) equipped with an extracorporeal circulation pump was used for blood and plasma circulation.

The DFPP conditions were set as follows: blood flow rate, 30 to 60 mL/min; plasma separation speed, 30% to 33% of the blood flow rate (plasma flow rate: 10 to 18 mL/min); and liquid waste flow rate, 1.5 to 3.0 mL/min. The target plasma volume was $1,500 \sim 2,000$ mL. Heparin was used as the anticoagulant.

The Plasmaflo OP-05W membrane-type plasma separator consisted of a hollow fiber made of polyethylene vinyl alcohol, with an internal diameter of 350 ± 50 μm, membrane thickness of 50 ± 10 μm, mean pore diameter of 0.3 μm, and membrane area of 0.5 m [2,3]. Permeability of plasma components (concentration in filtered plasma/concentration in original blood × 100) was not less than 95% for total protein, albumin, immunoglobulin, and total cholesterol at a blood flow speed of 80 to 130 mL/min and plasma flow rate of 20 to 44 mL/min. The Cascadeflo EC-50W membrane-type plasma separator consisted of a hollow fiber composed of an ethylene vinyl

alcohol co-polymer, with an internal diameter of 175 μm, membrane thickness of 40 μm, effective area of 2.0 m², and blood volume capacity of 150 ml. The instrument was filled with pyrogen-free sterile water before use.

Waste fluid treatment

For the analysis of DFPP waste fluids, two sample treatment methods were compared in this study: one in which physiological saline was added for washing and diluting samples prior to freezing, and the other in which waste fluids were directly frozen and then subjected to various treatments. In the first treatment method, waste fluid samples from the DFPP process were diluted and washed with 10 mL physiological saline, and were then kept frozen at -20°C until needed for analysis. Although the total fluid amount was not known for all waste fluid samples, the average fluid amount was approximately 100 mL, including the 10 mL of physiological saline. This value (100 mL) was therefore used when calculating the eliminated amount of AGEs in samples with unknown volumes. In the second treatment method, the DFPP waste fluid samples were directly frozen and then treated with either an ultrasonic homogenizer, acidification, alkalization, or the proteolytic enzymes porcine pancreatic elastase (Nacalai Tesque, Nakagyo-ku, Kyoto, Japan), keratinase (Wako Pure Chemical Industries, Ltd., Chuo-ku, Osaka, Japan), or collagenase (NittaGeratin Inc., Naniwa-ku, Osaka, Japan). The first method was used in this study.

Blood biochemistry analysis and measurement of AGEs, with the exception of N^{ε}-carboxymethyllysine (CML), in the waste fluid (n = 53) and serum samples (n = 16) collected from September 12, 2011 to March 26, 2012 were analyzed at Mitsubishi Medience (Minato-ku, Tokyo, Japan). N^{ε}-carboxymethyllysine (CML) in the waste fluid samples (n = 15) collected from April 2 to May 14, 2012 was analyzed by SRL, Inc. (Shinjuku-ku, Tokyo, Japan).

CML measurement

CML was measured by enzyme-linked immunosorbent assay (ELISA) using anti-CML antibody (Fushimi Farma, Marugame, Kagawa, Japan) as a primary antibody and horseradish peroxidase-labeled antibody (Fushimi Farma) as a secondary antibody for detection, as previously described [6,7].

Pentosidine measurement

Pentosidine levels in the serum and DFPP waste fluid samples were measured by high-performance liquid chromatography (HPLC) as reported previously [8]. Briefly, 50-μL sample aliquots were mixed with 1 mL of 6 N HCl and were then heated in a block incubator at 100°C for 18 h (acid hydrolysis). The HCl was then removed using a centrifugal evaporator, and the obtained residue was dissolved in 1 mL distilled water. A 500-μL aliquot of this solution was injected into a 3-mL ion exchange column (Oasis MCX; Waters, Milford, MA, USA), and 0.1 N HCl was then passed through the column. To elute pentosidine, 4.5 mL of 7% aqueous NH₃ was passed through the column. The eluate was evaporated to dryness and dissolved in 300 μL of a mixture of 10% acetonitrile and 0.2% heptafluorobutyric acid (HFBA). The target product was separated and quantified using reversed-phase HPLC with HFBA as an ion-pairing reagent. A standard curve was generated using known dilutions of pentosidine (PPL-FR, Strasbourg, France).

Malondialdehyde-modified LDL measurement

Malondialdehyde-modified LDL (MDA-LDL) was quantified using an MDA-LDL ELISA Kit (Sekisui Medical, Chuo-ku, Tokyo, Japan), as previously described [9-11]. Briefly, sample waste fluid and serum were treated with diluted waste fluid containing surfactant to change the structure of MDA-LDL. Solid-phase MDA-LDL was then combined with solid-phase anti-MDA-LDL mouse monoclonal antibody (ML25) in the well of 96-well microplates, and was then reacted with β-galactosidase-labeled anti-apo B mouse monoclonal antibody (AB16) to form solid-phase antibody/MDA-LDL/enzyme-labeled antibody immune complexes. Color development after the addition of o-nitrophenyl β-D-galactopyranoside was measured at an absorbance of 420 nm using a SPECTRA MAX 190 microplate reader (Molecular Devices, Tokyo, Japan), and the MDA-LDL concentration was then determined from standard curves generated from known quantities of MDA-LDL.

Statistical analysis

Data are expressed as means ± standard deviation. Changes in test values before and after DFPP were compared using the paired t-test or Wilcoxon test. All statistical analyses were performed with SPSS II software (IBM Japan, Minato-ku, Tokyo, Japan). The significance level was set at p < 0.05 for the two-sided test.

Ethical standards

The present study was conducted at the authors' clinic in compliance with the ethics principal outlined in the Helsinki Declaration and Personal Information Protection Act, with reference to the "Ministerial Ordinance on Good Clinical Practice" (Ordinance No. 28 of the Ministry of Health and Welfare (MHW), March 27, 1997). The study protocol was approved by our institutional ethics committee (Doshisha University), and all of the subjects provided written informed consent prior to participation in this study (Approval number #832).

Results

Reduction of serum AGEs and MDA-LDL by DFPP

The pentosidine concentrations in the serum samples collected before and after DFPP are presented in **Fig.1**. The amount of serum pentosidine significantly decreased from the baseline value of 121 ± 29 pmol/mL to 101 ± 21 pmol/mL after DFPP (p = 0.004), corresponding to a percentage removal of 14.6% ± 19.3% (n = 16). For samples in which pentosidine was lower than the detectable value of 35 pmol/mL, a concentration of 34 pmol/mL was used for analysis.

A similar analysis was performed to analyze the change in serum CML concentrations as a result of DFPP (**Fig.2**). DFPP significantly decreased serum CML from 6.7 ± 1.2 µg /mL to 4.4 ± 2.1 µg /mL (p < 0.001), corresponding to a percentage removal of 35.3% ± 30.0% (n = 15). For samples in which the CML level was lower than 1.2 µg/mL, a concentration of 1.0 µg/mL was used for the analysis.

The changes of serum MDA-LDL concentration after DFPP are presented in **Fig.3**. Serum MDA-LDL significantly decreased from 104.9 ± 49.2 to 65.8 ± 36.9 U/L (p < 0.001),

corresponding to a percentage removal of 35.8% ± 26.1% (n = 16). LDL-C was measured as a reference and changed from 104.3 ± 31.3 to 76.4 ± 17.5 mg/dl, corresponding to a percentage removal of 19.9% ± 35.3% (n = 16).
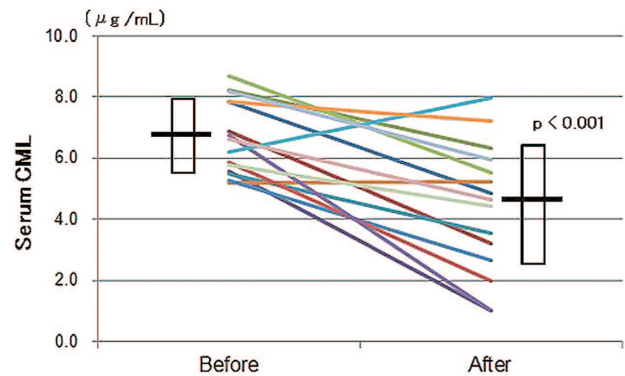
Treatment of DFPP waste fluids

Upon thawing of the waste fluid samples, the solutions appeared inhomogeneous due to the lack of pre-treatment
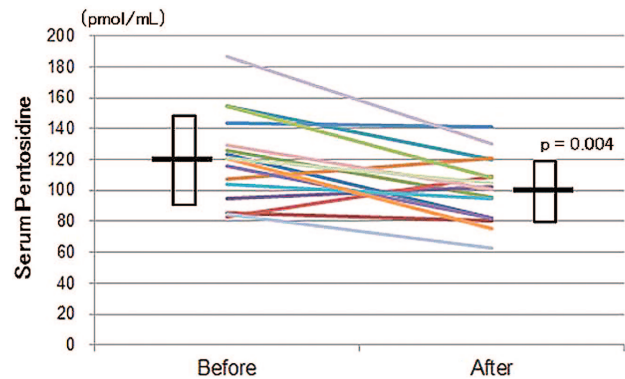
Fig.1. Change of serum pentosidine by DFPP.
Data are expresses as means ± standard deviation, n = 16, paired t test. DFPP: double filtration plasmapheresis.

Fig.2. Change of serum CML by DFPP.
Data are expressed as means ± standard deviation, n = 15, paired t test. DFPP: double filtration plasmapheresis, CML: N^ε-carboxymethyllysine.
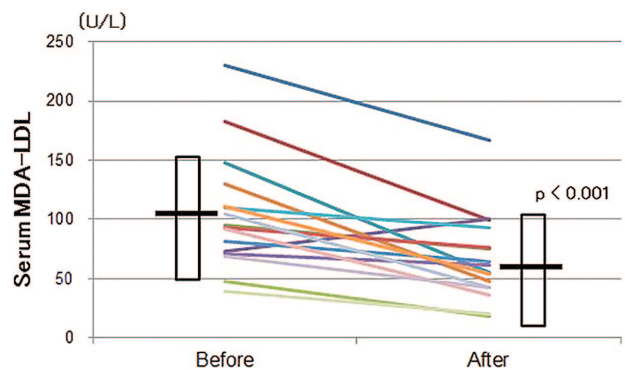
Fig.3. Change of serum MDA-LDL by DFPP.
Data are expressed as means ± standard deviation, n = 16, paired t test. DFPP: double filtration plasmapheresis, MDA-LDL: malondialdehyde-modified low-density lipoprotein.

before freezing. However, post-treatment of the thawed waste fluid samples with an ultrasonic homogenizer, or by acidification, alkalization, or proteolytic enzymes did not improve the homogeneity of the samples. In these samples, AGEs concentrations were lower than those prior to freezing. In particular, pentosidine was not detected in any of the thawed or pretreated samples. For this reason, we used the samples that were pretreated prior to freezing for further analyses.

Profile of DFPP waste fluids

The profiles of the DFPP waste fluids (n =53), which were comprised of approximately 10% physiological saline as a diluent, are shown in **Table 1**. For several of the samples, the concentrations of the investigated parameters were below the limit of detection of the assay and were therefore excluded from the analysis. The mean pentosidine concentration in the DFPP waste fluids was 60.7 ± 22.2 pmol/mL (n = 42), corresponding to an estimated removal amount of 6,000 pmol from 100 mL of waste fluid including physiological saline. For CML, the mean concentration in the waste fluids was 3.7 ± 1.7 μg/mL (n = 15), and the removal amount of CML from the total sample was estimated to be 370 μg (n = 15). The mean concentration of MDA-LDL in the waste fluids was 439.7 ± 398.8 U/L (n = 53), corresponding to an estimated removal amount of 44 U.

Table 1. Profiles of waste fluid from double-filtration plasmapheresis (DFPP).

Parameter	Mean ± S D	n
Total protein (g/dL)	4.4 ± 0.7	52
Albumin (g/dL)	2.0 ± 0.4	52
Glucose (mg/dL)	19.5 ± 16.7	40
TG (mg/dL)	365.3 ± 439.1	53
TC (mg/dL)	452.4 ± 202.2	53
HDL-C (mg/dL)	36.1 ± 17.7	52
LDL-C (mg/dL)	324.2 ± 159.2	52
MDA-LDL (U/L)	439.7 ± 398.8	53
CML (μg/mL)	3.7 ± 1.7	15
Pentosidine (pmol/mL)	68 ± 20	42

Waste fluid (average volume: approximately 100 mL) includes 10 mL physiological saline. Data are expressed as means ± standard deviation (SD). TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MDA-LDL, malondialdehyde-LDL; CML; N^{ε}-carboxymethyllysine.

Discussion

Removal of serum AGEs by DFPP

The measurement of serum AGEs and MDA-LDL before and after DFPP revealed that approximately 15% of pentosidine and 35% of CML and MDA-LDL were removed from serum by the DFPP process. The percent removal of CML and MDA-

LDL was similar to that found for LDL-C here (19.9%) and in our previous report (36.7%) [3]. The markedly lower efficiency for the removal of free pentosidine from serum may due to its lower molecular weight (MW 379) compared to CML (MW 494). Notably, however, the assay used to measure pentosidine was not limited to the free form, and it is possible that protein-bound pentosidine may be eliminated at similar levels to those of other AGEs such as CML. Although this possibility needs to be confirmed in future experiments, the present findings suggest that DFPP removes AGEs from serum, thus contributing to a reduction in glycative stress.

Treatment of DFPP waste fluids

For the analysis of DFPP waste fluids, two sample treatment methods were compared in this study: one in which physiological saline was added for washing and diluting samples prior to freezing, and the other in which waste fluids were directly frozen without any treatment. Upon thawing, the waste fluid samples mixed with saline appeared yellowish, semi-translucent, and uniformly homogenized, whereas the directly frozen samples were not homogeneous and contained white debris. Treatment of the latter samples by ultrasonic homogenization, acidification, alkalization, or enzymatic proteolysis, however, did not markedly improve the homogeneity of the samples.

The measured CML concentrations in the untreated samples were approximately 50% to 20% lower than those of the samples mixed with physiological saline prior to freezing, and pentosidine was also not detected in the untreated samples. These results indicate that AGEs have increased stability in DFPP waste fluid diluted with physiological saline.

MDA-modified LDL

MDA is a representative lipid peroxidation product that is capable of modifying apo B, a major LDL protein, forming MDA-LDL [9]. Evidence suggests that MDA-LDL and other modified LDLs are involved in the progression of arteriosclerosis and onset of coronary artery disease [10-13]. MDA-LDL has been identified as an oxidized form of LDL in many past studies [12,13]; however, the modification reaction was shown to occur during an early phase of the glycation reaction, which proceeds in three stages: an initial reaction, the generation of intermediate products, and a late reaction. Glycative stress is caused by compounds such as the reducing sugars glucose and fructose, which are formed at high concentrations during the post-prandial period in serum, and lipids that generate aldehyde and ketone groups through β-oxidation or peroxidation [1]. Thus, MDA-LDL can be considered a glycated form of LDL, and the elimination of serum MDA-LDL by DFPP, which reached approximately 35% in the present study subjects with hyper-LDL-cholesterolemia, may therefore lower glycative stress.

AGE intake and excretion

AGEs in foods are poorly digested and absorbed in the intestine, with only approximately 10% of AGEs reportedly being absorbed into the body [14,15]. AGEs are particularly abundant in fast food, as they are formed when meat is grilled at high temperature or in foods that are fried in oil [16,17]. However, certain types of AGEs contribute to the "umami" (savory) taste and have attractive flavors, whereas other AGEs function as anti-oxidants and have anti-glycation effects.

The daily dietary intake of CML typically ranges from 5.4

~ 11.3 mg [18], wiAssuming that 1.0 ~ 1.5 g creatinine/day is excreted in urine, the urinary excretion of CML is estimated to be 800 ~ 1,800 μg/day. As CML intake increases, the amount excreted in stool correspondingly increases; however, the urinary excretion of CML does not increase because CML excretion is dependent on renal function, for which creatinine excretion is a surrogate marker [18]. Although most (>90%) orally ingested CML remains in the stool without intestinal absorption [18], an increase in the CML intake amount would lead to higher total absorption without a concomitant change in urinary excretion and the possible accumulation of CML in the body.

In the present study, the amount of serum CML removed by DFPP was estimated to be 370 μg, which corresponds to 20% ~ 45% of daily urinary excretion. The relative amount of CML eliminated by DFPP would be higher in patients with renal dysfunction, who typically exhibited impaired AGE elimination. Thus, the use of DFPP for the removal of AGEs is expected to reduce glycative stress, particularly in patients with renal failure.

Conclusions

The study appears to have an elimination effect, and glycative stress reduction was presumption, as demonstrated here by the detection of the AGEs pentosidine and CML in high quantities in the DFPP waste fluids of subjects with hyper-LDL-cholesterolemia. Notably, the percent removal of CML was similar to that of LDL-C, whereas the removal efficiency of pentosidine was markedly lower, indicating that modifications to the DFPP process for the removal of low-molecular-weight AGEs, such as the combined use of a small-pore-size filter membrane or specific adsorbents, may be more effective for the reduction of glycative stress.

Acknowledgement

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