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RESEARCH ARTICLE

Effect of blood sample type on the measurement of advanced oxidation protein products as a biomarker of inflammation and oxidative stress in hemodialysis patients

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Abstract
Advanced oxidation protein products (AOPP) is widely used as a uremic biomarker, especially for cardiovascular disease. However, it has not been determined whether it is better to measure AOPP in plasma or serum. In this cross-sectional study, which included 102 patients undergoing maintenance hemodialysis, fibrinogen-free serum and defibrinated plasma samples were prepared. AOPP levels from fibrinogen-free samples displayed a stronger correlation with myeloperoxidase activity and levels of C-reactive protein, interleukin-6 and tumor necrosis factor-alpha, as well as prevalent cardiovascular disease, than AOPP levels obtained from plasma samples. These results demonstrated that fibrinogen interferes with the measurement of AOPP.

Keywords: *Advanced oxidation protein products; biomarker; cardiovascular disease; hemodialysis; sample preparation*

Introduction

Cardiovascular disease (CVD) remains the most common cause of excess morbidity and mortality in patients undergoing maintenance hemodialysis (MHD). However, the high frequency of cardiovascular events among these patients is disproportionate to conventional risk factor profiles (Kalantar-Zadeh et al.2003). As a consequence, considerable interest has been focused on "nontraditional" risk factors for CVD in the MHD population. Among the nontraditional risk factors examined, oxidative stress and elevated circulating levels of proinflammatory cytokines are speculated to play important roles (Stenvinkel et al.2005). These deleterious conditions are exacerbated by the recurrent activation of peripheral blood mononuclear cells (PBMCs) during hemodialysis (HD). In particular, interaction of the blood with the dialysis membrane may activate PBMCs, thereby causing the release of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), as well as myeloperoxidase (MPO).

MPO, which is an abundant mammalian phagocyte hemoprotein, might be a critical link between inflammation and oxidative stress (Himmelfarb.2004). Increased levels of MPO have been shown to be a risk factor for adverse cardiovascular events and correlate with the risk of mortality in uremic patients (Pecoits-Filho et al.2003, Nicholls & Hazen.2005, Kalantar-Zadeh et al.2006). Furthermore, MPO catalyzes the production of hypochlorous acid from hydrogen peroxide and chloride ions; hypochlorous acid is capable of oxidizing plasma proteins to generate advanced oxidation protein products (AOPP) (Witko-Sarsat et al.1996, Witko-Sarsat et al.1998).

The main component of AOPP is dityrosine-containing oxidized albumin (Capeillere-Blandin et al.2004); dityrosine is a highly fluorescent stable

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product of myeloperoxidase-induced oxidation (Marquez & Dunford.1995). By taking advantage of the linear absorbance of the dityrosine content at 340 nm (A_{340}), AOPP levels in blood can be conveniently measured by spectrophotometry (Witko-Sarsat et al.1996). AOPP themselves can activate inflammatory cells (Witko-Sarsat et al.1998, Capeillere-Blandin et al.2006) and contribute to the progression of CVD (Drueke et al.2002, Descamps-Latscha et al.2005, Liu et al.2006). Therefore, AOPP level is considered to be an economic biomarker of inflammation and oxidative stress and is used as such in many diseases, including diabetes mellitus, rheumatoid arthritis, and ulcerative colitis, especially for patients with uremia (Kalousova et al.2002, Abou-Seif & Youssef.2004, Baskol et al.2006, Kocak et al.2007, Baskol et al.2008). However, although spectrophotometry enables the AOPP level to be estimated easily, other components of blood might also contribute to the A_{340}. Unfortunately, the current assay protocol for AOPP has not been standardized. Both plasma and serum samples are used to measure AOPP (Kalousova et al.2002, Antolini et al.2005, Massy et al.2005, Thomas et al.2005, Krasniak et al.2007). The fact that the plasma AOPP level is higher than the serum level (Selmeci et al.2006, Valli et al.2007) results in confusion when results are compared among studies that use plasma and those that use serum to measure AOPP.

The main difference between plasma and serum is the content of fibrinogen, which is especially important in patients undergoing MHD because the fibrinogen level is elevated in such patients(Kaysen et al.2003). Oxidized fibrinogen contains dityrosine residues (Nowak et al.2007) and as a consequence fibrinogen contributes to the plasma A_{340} (Selmeci et al.2006). Therefore, it remains unclear whether the A_{340} of plasma, which contains fibrinogen, is equivalent to AOPP activity.

In this cross-sectional study, which included 102 patients on MHD, differences in AOPP values from plasma and fibrinogen-free samples were compared, and then the contributions of fibrinogen to the value of A_{340} were analyzed. To assess the implications of the use of different samples for the AOPP assay, the AOPP levels of different samples were analyzed as a biomarker of oxidative stress and prevalent CVD in patients on MHD.

Material and Methods

Study Population

For this cross-sectional study, patients who had received stable MHD treatment for at least 9 months were recruited from the Blood Purification Center, Guangdong General Hospital, China, between September 2008 and December 2008. They received dialysis three times a week for 4 h and fulfilled the criteria of dialysis adequacy (Kt/V more than 1.2). Blood pressure was measured before HD and classified according to the definitions provided by the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. Cardiovascular events were monitored every 3–6 months. Prevalent CVD was defined as a medical record of a myocardial infarction, angina, coronary artery bypass, angioplasty, stroke, peripheral arterial disease, or abdominal aortic aneurysm. The exclusion criteria included the manifestation of possible infection and antioxidant therapy or intravenous iron therapy 2 weeks before recruitment. In total, 56 patients with and 46 patients without prevalent CVD were enrolled. All 102 patients (49 men and 53 women; mean age 51 ± 12 years), were Han Chinese who had received HD treatment three times a week for 37 ± 16 months.

Control samples were selected randomly from 24 healthy subjects who visited our medical clinic for health screening (physical examination, electrocardiogram, chest X-ray, blood tests for liver and renal function, glucose and lipids). Conditions that were excluded were diabetes mellitus, dyslipidemia, hypertension, overt infection, and chronic kidney disease, which was defined as an estimated glomerular filtration rate (eGFR) of less than 60 mL/min per 1.73 m^2 in accordance with the modified eGFR equation for Chinese (Ma et al.2006).

The characteristics of the patients and controls are shown in Table 1.

Treatment of blood samples

Plasma and two types of fibrinogen-free sample, serum and defibrinated plasma, were prepared. Blood samples were collected from the MHD patients when routine clinical examinations were required. Predialysis blood samples were drawn directly from the dialysis tubing immediately after insertion of the needle into the vascular access and prior to the administration of heparin. The use of blood samples complied with the Declaration of Helsinki. Plasma and serum samples were collected in plasma separator tubes containing anticoagulant ethylenediamine tetraacetic acid (EDTA) or serum separator tubes containing clot activator (BD, Franklin Lakes, NJ, USA), respectively. Serum had to be kept at room temperature for at least 30 minutes for clotting. Defibrinated plasma was prepared by the thrombin method as described previously (Gaffney & Wong.1992): human thrombin (RAAS Blood Products Co., Ltd. Shanghai, China) was dissolved in 0.90 M calcium chloride solution (500 IU/mL) and added to plasma at a ratio of 1:4 (thrombin solution:plasma, v:v). After incubating at 37 °C for 2 hours, the samples were centrifuged at 30,000×g at 4 °C for 10 minutes, then the supernatant was collected and used as defibrinated plasma. We have confirmed that

Table 1. Characteristics of the study population.

	Health controls (n = 24)	Patients without CVD (n = 46)	Patients with CVD (n = 56)
Age	49 ± 8	51 ± 12	51 ± 13
Male, n (%)	12 (50)	31 (30.4)	27 (26.5)
Albumin (g/L)	42 (40, 44) *	32 (29, 35) #	36 (32, 38)
Hemoglobin (g/L)	131 ± 11*	115 ± 13#	104 ± 12
CRP (mg/L)	4.0 ± 0.9*	4.8 ± 1.2#	6.2 ± 1.5
MPO activity (U/L)	60.3 ± 10.9*	132.8 ± 55.6 #	236.6 ± 58.3
Plasma AOPP(μmol/L)	26 (22, 30) *	122 (98, 143) #	146 (126, 169)
Defibrinated plasma AOPP(μmol/L)	21 (18, 27) *	71 (58, 83) #	124 (101, 143)
Serum AOPP(μmol/L)	21 (16, 24) *	63 (55, 74) #	100 (88, 128)
IL-6 (pg/ml)	13.4(11.0-15.3) *	20.8 (12.2, 28.5) #	29.8 (19.0-32.6)
TNF-α (pg/ml)	6.6 ± 1.2*	8.9 ± 2.4#	11.1 ± 2.7
Fibrinogen (g/L)	3.8 ± 0.9*	5.2 ± 2.1	5.9 ± 2.5
Diabetes mellitus, n (%)		11 (23.9) #	44 (78.6)
BP class		1 (0, 1) #	2 (1, 3)
HD vintage (months)		32 (22, 39)	40 (24, 52)
Biocompatible dialyzer, n (%)		34 (73.9) #	26 (46.4)
PTH (pg/mL)		235 (202, 298) #	360 (289, 429)

*P<0.05, controls versus patients undergoing hemodialysis; # P<0.05, patients with cardiovascular disease versus those without cardiovascular disease

CVD: cardiovascular disease; CRP: C reactive protein; MPO: myeloperoxidase; AOPP: advanced oxidation protein products; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha; BP class: blood pressure classified by JNC-VI; HD: hemodialysis; PTH: intact parathyroid hormone.

the EDTA, clot activator, and thrombin solution had no effect on A_{340} (data not shown).

Recently, it has been demonstrated that turbidity induced by triglycerides can cause the AOPP level to be overestimated and that delipidation before the analysis yields a lower AOPP value that reflects oxidative stress more accurately (Valli et al.2007). Therefore, the blood samples were centrifuged (10,000×*g*, 1 hour, 4 ºC) to separate the lipids, and samples for the measurement of AOPP were taken from below the lipid layer (Li et al.2007).

Measurement of AOPP

All tests were performed at 25 °C. AOPP values were determined in plasma, defibrinated plasma, and serum samples as described previously (Witko-Sarsat et al.1996, Witko-Sarsat et al.1998). Briefly, 200 μL of the test samples were diluted 1:5 in PBS and mixed with 20 μL of acetic acid. In the wells for the standard curve, 10 μL of potassium iodide (Sigma, St Louis, USA) were added to 200 μL of chloramine-T solution (Sigma, St Louis, USA) followed by 20 μL of acetic acid. The A_{340} of the reaction mixture was analyzed in a microplate reader (Thermo Multiskan MK3, Vantaa, Finland) within 3 min of adding the acetic acid. AOPP levels were expressed as μmol/L of chloramine-T equivalents.

Biochemical measurements

Plasma levels of fibrinogen were quantified by the Von Clauss method (Diagnostica Stago, Asnieres-sur-Seine, France). Serum C-reactive protein (CRP) was determined by an immunoturbidimetric assay (Beckman, USA). Plasma was used for the measurement of circulating levels of IL-6 and TNF-α and myeloperoxidase activity. Levels of IL-6 and TNF-α were determined by using human ELISA kits (Jingmei Biotech, Shanghai, China). Myeloperoxidase activity was assessed using an MPO Detection Kit (Jiancheng Bioengineering Institute, Nanjing, China). This method is based on the MPO-catalyzed hydrogen peroxide-mediated oxidation of o-dianisidine. The absorbance of the yellow oxidation product was measured at 460 nm. One MPO activity unit was defined as the amount of enzyme that degraded 1 μmol peroxide per min at 25 °C (Bradley et al.1982, Yazici et al.2004).

Statistical analysis

The data were expressed as mean values ± SD or medians (25th, 75th percentile) and analyzed using SPSS software (version 13.0, Chicago, IL). Student's t-test or the Mann–Whitney U-test was performed, as appropriate. To compare AOPP levels from different samples, Friedman's test was used. When a global significant difference existed, post hoc multiple comparison procedures were performed. Spearman's correlation coefficient (r_s) was used to assess the correlation between two variables. We evaluated the cardiovascular risk by determining the odds ratio (OR) in both univariate and multivariate binary logistic regression models. Variables studied in the multivariate models included age, gender, diabetes mellitus (yes/no), blood pressure class (0–3), dialysis vintage

(number of months on MHD therapy), biocompatibility of dialyzer (yes/no), and levels of blood hemoglobin, serum albumin, intact parathyroid hormone and AOPP (each 20 μmol/L increase). To compare the discrimination values of the AOPP levels from each blood sample for prevalent CVD, the receiver operating characteristic (ROC) curve was plotted and the areas under the ROC curve were calculated. Two-tailed tests were performed for all comparisons, and a P value < 0.05 was considered to be statistically significant.

Results

The relationship of AOPP levels among the three types of sample

Among the 102 patients undergoing MHD, the AOPP levels in plasma, defibrinated plasma, and serum were 135 (109, 160) μmol/L, 98 (71, 133) μmol/L, and 82 (61, 109) μmol/L, respectively. Although the differences between these values were statistically significant (all P values < 0.001), there were correlations between the pairs of AOPP values (plasma to defibrinated plasma, $r_s = 0.760$; plasma to serum, $r_s = 0.701$; defibrinated plasma to serum, $r_s = 0.907$; all P values < 0.001).

AOPP as a marker of inflammation and oxidative stress

AOPP levels in plasma, defibrinated plasma, and serum all correlated with serum MPO activity, and levels of CRP, IL-6, and TNF-α, and only the correlation between plasma AOPP and the CRP level was not significant. Furthermore, the AOPP levels in the fibrinogen-free samples had a higher correlation coefficient with the markers of inflammation and oxidative stress than the plasma AOPP levels (Table 2).

Table 2. Comparison of Spearman correlation coefficients (r_s) of AOPP with 4 measures of inflammation and oxidative stress.

Variables	MPO activity	CRP	IL-6	TNF-α
Plasma AOPP	0.512**	0.147	0.241*	0.200*
Defibrinated plasma AOPP	0.731**	0.278**	0.389**	0.340**
Serum AOPP	0.725**	0.300**	0.307**	0.279**

*P < 0.05; **P < 0.01

Effect of fibrinogen A_{340}

From the two types of fibrinogen-free sample, we were able to calculate the contribution of plasma fibrinogen to A_{340} (plasma fib-A_{340}) by determining the difference between the AOPP values from the plasma and fibrinogen-free samples. The plasma fib-A_{340} value had a weak negative or nonsignificant relationship with markers of inflammation and oxidative stress (Table 3).

AOPP as a cardiovascular biomarker

AOPP levels from the fibrinogen-free samples were associated with increased risk for prevalent CVD. In contrast, plasma fib-A_{340} seemed to have a negative relationship with prevalent CVD (Table 4). This was supported by the finding that the AOPP level in plasma, which included the effect of plasma fibrinogen, had a lower OR with prevalent CVD than the AOPP level from the fibrinogen-free samples (Table 4).

Furthermore, AOPP levels from the plasma, defibrinated plasma, and serum samples yielded an area under the ROC curve of 0.80 (95% confidence interval 0.73 to 0.88), 0.92 (95% confidence interval 0.87 to 0.97), and 0.90 (95% confidence interval 0.85 to 0.96), respectively, in discriminating prevalent CVD in MHD patients (Figure 1).

Table 3. Comparison of Spearman correlation coefficients (r_s) of A_{340} of plasma fibrinogen with 4 measures of inflammation and oxidative stress.

Variables	MPO activity	CRP	IL-6	TNF-α
Plasma fib-A_{340}a	-0.376**	-0.214**	-0.257**	-0.228*
Plasma fib-A_{340}b	-0.226*	-0.164	-0.113	-0.065

Plasma fib-A_{340}a: difference of the AOPP value from plasma and defibrinated plasma; Plasma fib-A_{340}b: difference of the AOPP value from plasma and serum.
*P < 0.05; **P < 0.01

Table 4. Increased risk for prevalent cardiovascular disease of increased AOPP levels (each 20μmol/L increase) from 5 contents contributing to blood A_{340}.

	Univariate model		Multivariate model	
	OR (95%CI)	P value	OR (95%CI)	P value
Plasma AOPP	1.41 (1.12-1.77)	0.003	1.66 (0.94-2.93)	0.080
Defibrinated plasma AOPP	2.68 (1.84-3.90)	<0.001	3.68 (1.42-9.51)	0.007
Serum AOPP	2.56 (1.72-3.80)	<0.001	2.69 (1.28-5.65)	0.009
Plasma fib-A_{340}a	0.52 (0.37-0.73)	<0.001	0.38 (0.18-0.82)	0.013
Plasma fib-A_{340}b	0.77 (0.59-1.00)	0.050	0.73 (0.41-1.32)	0.300

Plasma fib-A_{340}a: difference of the AOPP value from plasma and defibrinated plasma; Plasma fib-A_{340}b: difference of the AOPP value from plasma and serum.

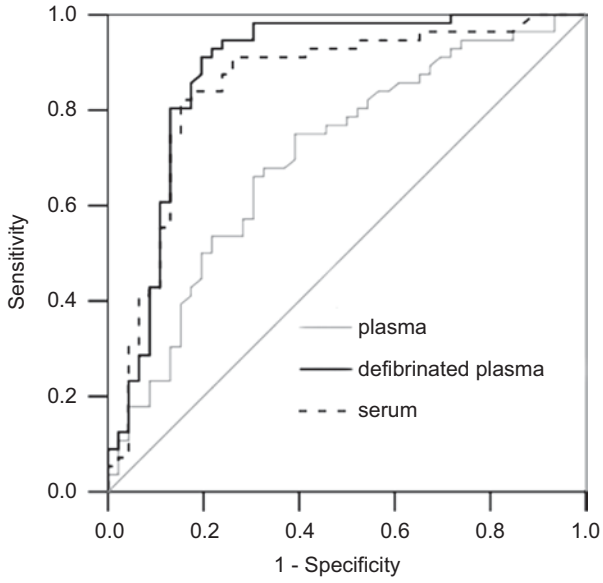

Figure 1. Discrimination value of AOPP levels from different samples for prevalent cardiovascular disease

Discussion

The present study provides several lines of evidence that suggest that plasma fibrinogen is not a component of AOPP but rather interferes with the measurement of AOPP.

The AOPP levels measured in plasma were significantly higher than those measured in fibrinogen-free blood samples, which confirmed that fibrinogen did contribute to the plasma A_{340}. This observation agreed with a previous study by Selmeci et al., in which the AOPP level in serum was 32.5% of that in EDTA-anticoagulated plasma (Selmeci et al.2006). To obtain fibrinogen-free blood samples, Selmeci et al. used a heating method in which plasma fibrinogen is removed by heating at 50 °C, whereas native albumin is retained. By using this method, they found that a decrease in plasma A_{340} paralleled the decrease in fibrinogen. However, it remained unresolved whether plasma A_{340} was an accurate measure of AOPP activity.

To resolve this issue, we prepared two types of fibrinogen-free blood samples, serum and defibrinated plasma, by clotting methods. The results showed that plasma fib-A_{340} had no relationship, or even a negative association, with the level of CRP, IL-6 or TNF-α, as well as MPO activity, whereas AOPP levels in the fibrinogen-free samples were correlated more strongly with the indices of inflammation and oxidative stress than those in plasma (Table 2 and Table 3). Furthermore, plasma fib-A_{340} was not a cardiovascular risk factor and AOPP levels from fibrinogen-free blood samples were a better indicator of the risk of prevalent CVD than AOPP levels from plasma (Table 4). These results were supported by the definition of AOPP, which states that AOPP are cross-linked oxidized proteins that contain dityrosine and carbonyl groups and arise from MPO-related oxidation (Witko-Sarsat et al.1996). Although plasma fibrinogen was found to be much more susceptible to carbonyl oxidation than albumin in an iron/ascorbate hydroxyl radical generating system (Shacter et al.1994), plasma albumin rather than fibrinogen is the major target of carbonyl oxidation in uremia (Himmelfarb & McMonagle.2001, Ding et al.2006). A possible explanation for these different results is that MPO-catalyzed oxidative reactions and other pathways are more significant than metal-catalyzed oxidation in MHD patients, and the two oxidative pathways have different effects on different proteins. Taken together, we could conclude that the A_{340} of plasma fibrinogen did not represent AOPP activity, which might reduce the discriminatory value of plasma AOPP as a cardiovascular biomarker in MHD patients.

The AOPP level in serum was a little lower than that in defibrinated plasma, which suggests that some components that contribute to A_{340} are lost during the clotting process for whole blood. However, there was a significant correlation between AOPP levels in serum and in defibrinated plasma ($r_s = 0.907$, $P < 0.001$) in patients on MHD. Most importantly, elevated serum AOPP levels had a similar relationship with prevalent CVD and showed a similar area under the ROC curve as compared with defibrinated plasma AOPP levels. Given that the simplest method to eliminate fibrinogen is to generate serum, the use of serum samples is a promising approach for the measurement of AOPP in terms of convenience.

Multiple factors are involved in the high risk of CVD in patients undergoing MHD. Besides classic risk factors such as hypertension and prevalence of diabetes mellitus, the patients in this study with prevalent CVD presented with prominent malnutrition, inflammation, and oxidative stress. The patients on MHD also had a higher level of plasma fibrinogen than the controls (Table 1). However, the difference in plasma fibrinogen level between the patients with and without CVD was not significant, which differed from the results of a previous study in which elevated plasma fibrinogen correlated with CVD in hemodialysis patients (Koch et al.1997). This discrepancy might be explained by the weakness of the association between fibrinogen and prevalent CVD (Irish.1998). Thus, fibrinogen levels alone could not discriminate prevalent CVD in this study with limited sample size. Moreover, the small sample size limited the number of variables that could be controlled and a type II error should be considered. Therefore, it cannot be concluded that AOPP is not a uremic biomarker because positive results obtained in previous studies were based on plasma AOPP and

plasma AOPP was not an independent risk factor for CVD as shown by multiple analyses in the present study ($P = 0.080$). On the contrary, our study confirmed the reliability of AOPP as a cardiovascular biomarker in patients on MHD, and suggested that AOPP would have better clinical value if the effect of fibrinogen is removed.

Another possible confounding factor that we did not examine is the presence of fibrinogen fragments (FF), which are present in both uremic plasma and serum, but are usually absent in the circulation of healthy individuals (Kozek-Langenecker et al.1999, Kaplan et al.2003, Thekkedath et al.2006). With respect to the A_{340} of fibrinogen, FF might contribute to the plasma A_{340}. However, the level of FF in plasma (1.14 ± 0.85 g/L) is much lower than that of plasma fibrinogen (Thekkedath et al.2006). Furthermore, given that dityrosine linkages may be degraded during the lysis of fibrinogen, the A_{340} of FF might be not as important as that of fibrinogen. Moreover, it is much easier to obtain plasma or serum samples during clinical practice than FF-free blood samples. As a consequence, given that the main difference between plasma and serum is not FF, we did not investigate the possible effects of FF in the present study. The results indicated that serum samples that contain FF can provide an effective means of measuring the biomarker AOPP in a cost-efficient manner.

Other limitations of this study include the restrictions of a cross-sectional study: the present study did not investigate the causality of elevated AOPP levels and increased cardiovascular risk in patients on MHD. As a consequence, a prognostic role for AOPP with respect to the occurrence of CVD should be investigated further in a future cohort study. In addition, AOPP is widely used as a marker in other conditions in which the index of MPO-related oxidative stress is not as strong as in patients on MHD. Therefore, the relationship of AOPP levels to other pathological conditions also needs to be investigated.

In summary, our work provides evidence that fibrinogen in plasma interferes with the measurement of AOPP and that serum AOPP levels are more appropriate than plasma AOPP levels as a biomarker of inflammation, oxidative stress, and cardiovascular risk in patients undergoing MHD.

Declaration of interest

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