Journal of Clinical Medicine and Research Vol. 3(8), pp. 114-119, December 2011
Available online http://www.academicjournals.org/JCMR
ISSN 2141-2235 ©2011 Academic Journals
DOI: 10.5897/JCMR11.053

Full Length Research Paper

The serum levels of P-cresol and Indoxyl sulfate in different hemodialysis vintage

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Accepted 25 October, 2011

Indoxyl sulfate (IS) and para-cresol (p-cresol) belong to the group of protein-bound uremic toxins that are poorly cleared by dialysis and are associated with poor clinical outcomes. The goal of our study was to evaluate the relationship between dialysis time and serum levels of IS and *p*-cresol in hemodialysis (HD) patients. Our study enrolled 96 stable HD patients who were categorized into 8 subgroups on the basis of the duration for which they received dialysis within a period of 1 year. Patients with acute infection, malignancy or those who were younger than 18 years of age were excluded. Serum levels of total and free p-cresol and indoxyl sulfate were measured using ultra performance liquid chromatography (UPLC). Biochemical data was also collected concurrently. In our study, we found that only unbound IS and total IS were significantly and positively correlated with the dialysis time in HD patients by trend analysis (p= 0.020 and p=0.007, respectively). Other independent variables, including the levels of unbound p-cresol, total p-cresol, hemoglobin, highly sensitive C-reactive protein (CRP), Kt/V and albumin, did not show significant correlation with the length of HD treatment. Our results inferred that patients undergoing longer hemodialsysis may lead to higher serum levels of IS.

Key words: Hemodialysis time, indoxyl sulfate, p-cresol, p-cresyl sulfate and protein-bound uremic toxin.

INTRODUCTION

Uremic retention solutes accumulate in patients with chronic kidney disease (CKD). These molecules may deteriorate the biochemical and physiological dysfunctions that define the uremic syndrome (Vanholder et al., 1999). There are three major groups of uremic toxins based upon their chemical and physical characteristics including small water-soluble compounds, protein-bound compounds and larger middle-molecules (Vanholder et al., 2003). Earlier studies concentrated on small water soluble and large middle molecules (PHeimbürger et al., 1997; MacAllister et al., 1996). However, the focus of the clinicians is shifting toward protein-bound uremic toxins recently.

Protein-bound uremic toxins, including at least 25 kinds of different toxins, have been neglected for a long time and its clinical importance has been demonstrated in recent years. Para-cresol (p-cresol) and indoxyl sulfate (IS) belong to the group of protein-bound uremic toxins and accumulate as renal function declines (Vanholder et

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al., 2003).

In vivo, p-cresol exists predominantly as conjugated *p*-cresyl sulfate (PCS), p-cresylglucuronate and unconjugated *p*-cresol are not detectable (De Loor et al., 2005; Martinez et al., 2005). However, previous reports demonstrated that various toxic effects of p-cresol in *vitro* (Wratten et al., 1999; Vanholder et al., 1995; Dou et al., 2002). Their emphasis was placed on *p*-cresol since the determination methods were based on deproteinization by acidification, leading to the disintegration of conjugates by hydrolysis (De Smet et al., 2003). One study suggested that p-cresylsulfate stimulated baseline leukocyte activity, whereas the p-cresol essentially inhibits activated leukocyte function (Schepers et al., 2007). Recently, free p-cresol concentrations also have been reported to be associated with the poor clinical outcomes in hemodialysis (HD) patients (Bammens et al., 2006; Meijers et al., 2008; Lin et al., 2010).

However, administration of IS in an animal study has been reported to result in decreased renal function and increased glomerular sclerosis (Niwa et al., 1997). IS also induced oxidative stress in endothelial cell and play a role in inhibition of endothelial proliferation and wound repair (Dou et al., 2004). IS was regarded to be involved in the pathogenesis of atherosclerosis in dialysis patients (Taki et al., 2007). These findings show the importance of IS in HD patients. Both of the toxins originated from the metabolism of amino acids by the intestinal flora and are strongly protein-bound (Niwa et al., 1981; Hida et al., 1996). The current dialysis technique could not remove the two toxins effectively. Thus, our study objective was to evaluate the relationship between serum levels of p-cresol and IS and dialysis time.

MATERIALS AND METHODS

Subjects

Our study comprised 96 stable HD patients, aged 30 to 80 years, from June to July 2007 in a single medical center. All patients were categorized into 8 subgroups (n= 15, 15, 12, 12, 11, 12, 10 and 9 respectively) on the basis of the duration for which they received dialysis within a period of 1 year. Patients with acute infection or malignancy and those aged less than 18 years were excluded from the study. The cause of end-stage renal failure in the total number of patients was type 2 diabetic nephropathy (n = 45), chronic glomerulonephritis (n = 43), polycystic kidney disease (n = 6), or lupus nephritis (n = 2).

All study patients were on 4 h maintenance dialysis three times a week for 6 to 120 months using a synthetic dialysis membrane. The dialyzers were not reused for all patients. Dialysis efficiency was evaluated according to the kidney disease outcomes quality initiative (KDOQI) guidelines, and single-pool Kt/V of urea nitrogen was calculated (Daugirdas, 1989). Residual renal function was estimated from an interdialytic urine collection and expressed as weekly renal Kt/V (rKt/V). The normalized protein catabolic rate (nPCR; g/kg/day) was calculated as a measure of the daily protein intake. 42 patients underwent conventional dialysis with GAMBRO artificial kidney (18 polyflux 14 L and 24 polyflux 17L), and 54 underwent high flux dialysis with Fresenius medical care artificial kidney (30 F80S and 24 F100S). Patient characteristics and biochemical parameters, including age, gender, intact-PTH (i-PTH), calcium, phosphate, and highly sensitive C-reactive protein (hs CRP), and alkaline phosphate (Alk-p), were examined concurrently.

This study was performed based on the Declaration of Helsinki Principles and approved by the ethics committees of the Mackay Memorial Hospital. All study patients have signed informed consent.

Laboratory assessment

At inclusion, blood samples of patients were taken immediately before the HD session two times a week (second and third session). All serum free and total p-cresol (mg/L) and IS (mg/L) were measured 2 times a week to obtain an average value. Other biochemistries including hemoglobin (g/dL), calcium (mg/dL), phosphate (mg/dL), bicarbonate (mmol/L), i-PTH (pg/mL), albumin (g/dL), urea (mg/dL), creatinine (mg/dL) were measured before the second HD session during the same week.

The bromocresol green method was used for the determination of albumin. The serum levels of hs-CRP were measured using a Behring Nephelometer II (Dade Behring, Tokyo, Japan). Serum p-cresol and IS (that is, combined free and protein-bound fractions) were analyzed with ultra performance liquid chromatography (UPLC). Briefly, serum samples were deproteinized by the addition of three parts methanol to one part serum for determination of IS. Total p-cresol (that is, the combined free and protein-bound fraction) was analyzed after deproteinization (acid and heat) and extraction (ethyl acetate) of serum samples. The free concen-trations of IS and p-cresol were measured in serum ultrafiltrates obtained using Microcon YM-30 separators (Millipore, Billerica, MA, USA), followed by the same sample preparation and analysis that was done for serum p-cresol and IS.

A UPLC assay using detection at the spectra of 280 nm of the PDA detector was performed at room temperature on an ACQUITY UPLC® BEH phenyl column of 2.1 × 100 mm. Buffer flow was 0.4 mL/min using 10 mM $NH_4H_2PO_4$ (pH = 4.0) (A) and 100% acetonitrile (B) with a gradient from 82.5%A/17.5%B to 55%A/45%B, over 9 min.

Under these conditions, IS and p-cresol appeared at 1.4 and 2.75 min, respectively. Standard curves from IS and p-cresol at 0.5, 1, 2.5, 5, and 10 mg/L, processed in the same manner as the serum samples, correlated to the serum samples with average r^2 values of 0.999 ± 0.001. Quantitative results were obtained and calculated as concentrations (mg/L). The sensitivity of this assay was 0.225 mg/L for IS and 0.425 mg/L for p-cresol.

Statistical analysis

All continuous results were expressed as the mean ± standard deviation (SD). The relationship between free IS, total IS, free p-cresol, total p-cresol, hsCRP, Hb, albumin, Kt/V, rKt/V, body weight, mean UF (ultrafiltration), sex, gender and dialysis length were analyzed by univariate and multivariate linear regression analysis (method: stepwise) and trend analysis. A value of *P* less than 0.05 was considered statistically significant. The statistical analysis was conducted by using SigmaStat for Windows, version 2.03 (SPSS Inc., Chicago, IL, USA).

RESULTS

Of the 96 study participants (mean age, 62.61 ± 8.12 year; 56 men and 40 women), 83 (86.45%) had hypertension; 45 (46.87%), diabetes; 43 (44.80%), chronic glomerulonephritis (cGN); 6 (6.25%), polycystic kidney disease; 2 (2.08%), lupus nephritis. The patients

Table 1. Baseline characteristics of the patients.

Patient demographics	HD patients (n = 96)
Median age (y)	62.61 ± 8.12
Men	56 (58.33%)
Women	40 (41.67%)
Time of HD (m)	40.27 ± 23.47
Diabetes	45 (46.87%)
PKD	6 (6.25%)
SLE	2 (2.08%)
cGN	43 (44.80%)
HTN	83 (86.45%)
SBP (mmHg)	145.47 ± 16.08
DBP (mmHg)	85.47 ± 7.27
nPCR (g/kg/day)	1.20 ± 0.11
Kt/V	1.66 ± 0.24
rK/V	0.05 ± 0.09
CO_2 (mmol/L)	22.18 ± 1.75
Hb (g/dL)	10.44 ± 1.14
Creatinine (mg/dl)	10.85 ± 2.04
Hct (%)	30.86 ± 3.38
Albumin (g/dL)	4.05 ± 0.27
Calcium (mg/dL)	8.88 ± 0.59
Phosphate (mg/dL)	5.20 ± 1.02
I-PTH (pg/mL)	331.08 ± 186.97
Alk-P (IU/L)	100.01 ± 33.64
hs CRP (mg/dL)	0.75 ± 0.68

were divided into 8 subgroups depending on the duration of HD treatment. The mean SBP was 145.47 ± 16.08; DBP, 85.47 ± 7.27; duration of HD treatment, 40.27 ± 23.47; serum Kt/V value, 1.66 ± 0.24; nPCR, 1.20 ± 0.11 g/kg/day; bicarbonate, 22.18 ± 1.75 mmol/l; hemoglobin (Hb), 10.44 ± 1.14 g/dl; hematocrit (Hct), 30.86 ± 3.38%; creatinine, 10.85 ± 2.04 mg/dl; albumin, 4.05 ± 0.27 g/dl; calcium, 8.88 ± 0.59 mg/dl; phosphate, 5.20 ± 1.02 mg/dl; intact parathyroid hormone (i-PTH), 331.08 ± 186.97 pg/ml; alkaline phosphatase (Alk-P), 100.01 ± 33.64 IU/l; and high-sensitivity C reactive protein (hs-CRP), 0.75 ± 0.68 mg/dl (Table 1).

Table 2 shows the results of the trend analysis performed to investigate the correlation of IS and *p*-cresol with the duration of HD treatment. For each group, the interval of dialysis was 1 year. We found that only unbound IS and total IS were positively correlated with the duration of HD ($p = 0.020$ and 0.007, respectively). Other variables, including the levels of unbound *p*-cresol, total *p*-cresol, Hb, Kt/V, rKt/V, hsCRP and albumin, did not show a significant correlation with the duration of HD treatment. In addition, we also used regression analysis to investigate the relationship between independent variables including factors mentioned above, sex, gender, body weight, UF per session and dialysis length. After adjusting confounding factors by multivariate analysis, only total IS had significant correlation with dialysis time ($p < 0.05$). Figure 1 show the changes in the serum concentrations of unbound / total IS and *p*-cresol with different durations of dialysis treatment. Our results revealed that dialysis patients with longer duration of dialysis had higher serum free and total IS.

DISCUSSION

In this study, we found that the serum level of IS but not that of *p*-cresol was correlated with the dialysis vintage. From the results, we inferred that in contrast to short-term HD, long-term HD results in the accumulation of unbound and total IS in the serum.

Protein-bound uremic toxins, especially IS and p-cresol have been reported to play an important role in CKD and dialysis patients. Both of IS and *p*-cresol are produced during the amino acid metabolism by intestinal flora and bind strongly to proteins. Previous vitro studies have shown that *p*-cresol has various toxic effects: It inhibits the synthesis of platelet-activating factor (Wratten et al., 1999), reduces responses elicited by activated polymorphonuclear cells (Vanholder et al., 1995), and suppresses the responses of endothelial cells to inflammatory cytokines (Dou et al., 2002). One study

Table 2. The relationship between IS/p-cresol, Hb, albumin and different time to HD.

Variable	Different time course of hemodialysis (years)								
	<1 (n = 15)	1-2 (n=15)	2-3 (n=12)	3-4 (n=12)	4-5 (n=11)	5-6 (n=12)	6-7 (n=10)	>7 (n = 9)	p
Free IS (mg/L)	3.5±1.9	3.8±3.2	4.9±3.5	3.7±2.0	4.3±1.5	5.7±4.3	5.7±2.7	5.1±2.7	0.020
Free PC (mg\L)	1.4±1.4	1.5±1.4	1.5±0.9	1.6±1.0	1.1±0.6	1.4±0.8	2.5±1.5	1.7±0.9	NS
Total IS (mg/L)	3.29±13.7	35.8±18.6	41.5±16.5	37.7±15.6	50.5±11.9	47.0±17.1	48.1±17.0	41.0±10.7	0.007
Total PC (mg/L)	20.0±13.7	20.0±13.8	22.4±11.9	25.8±13.9	18.0±8.7	20.0±12.2	27.2±12.7	20.7±14.1	NS
Free IS/Total PC	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.1	NS
Free PC/Total PC	0.1±0.0	0.1±00	0.0±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	NS
Hb (g/dL)	10.1±1.4	10.3±1.6	10.9±1.7	10.4±1.5	10.6±1.2	11.0±1.2	9.7±1.7	11.2±2.1	NS
hsCRP(mg/dL)	0.4±02	0.6±0.3	0.5±0.2	0.4±0.1	0.7±0.3	0.5±0.2	0.6±0.2	0.5±0.3	NS
kt/V	1.3±0.2	1.5±0.2	1.6±0.2	1.5±0.1	1.5±0.2	1.6±0.1	1.4±0.1	1.5±0.2	NS
rKt/V	0.22±0.11	0.07±0.04	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	NS
Albumin (g/dL)	3.9±0.3	4.0±0.3	4.1±0.3	0.3±4.1	4.3±0.3	4.1±3.0	41±0.3	4.1±0.1	NS

Figure1. The time course of serum free/total IS and p-cresol in different dialysis groups based on dialysis time. (The results were expressed as the median +/- IQR/2)

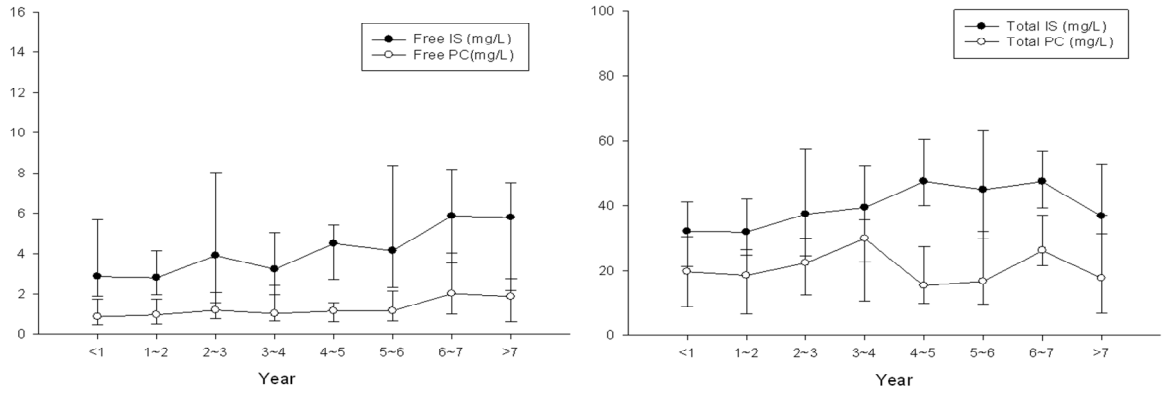

Figure 1. The time course of serum free/total IS and p-cresol in different dialysis groups based on dialysis time. (The results were expressed as the median +/- IQR/2).

have revealed that the concentration of unbound *p*-cresol is correlated with mortality in dialysis patients (Bammens et al., 2006) and is a risk factor for cardiovascular disease in non-diabetic HD patients (Meijers et al., 2008). In our previous study, we found that the serum free p-cresol and hsCRP were closely associated with the infection related hospitalization in HD patients after 20-month follow-up (Lin et al., 2010).

Further, the serum concentration of IS was reported to be related with the progression of chronic kidney disease (CKD) (Niwa et al., 1997). In addition, an *in vitro* study has shown that IS will lead to endothelial dysfunction (Dou et al., 2004). Taki et al. (2007) suggested that IS might be involved in the pathogenesis of atherosclerosis in dialysis patients. Thus, elevated serum concentrations of IS and *p*-cresol may lead to adverse outcomes in HD patients.

Accordingly, many clinicians are interested in determining the factors that influence the serum concentrations of these 2 toxins. One recent study demonstrated that the two toxins were accumulated in advanced CKD patients (Lin et al., 2011). For HD patients, the duration of HD treatment may be one important factor. Because of the high affinity of IS and *p*-cresol for serum albumin, these toxins cannot be effectively eliminated with regular dialysis. Bammens et al. (2004) suggested that patients with convective dialysis procedure had lower p-cresol level that those with high

flux dialysis (Bammens et al., 2004). Recently, one study reported that neither HD nor HDF can effectively remove serum p-cresylsulfat and IS (Krieter et al., 2010). In addition, another issue affecting serum protein-bound uremic toxins is that the residual renal function (RRF) in dialysis patients. RRF had been considered as an important factor of clinical outcome in hemodialysis or peritoneal dialysis patients (Shemin et al., 2001; 2000). Studies indicated that RRF played an important role in clearance of p-cresol in peritoneal dialysis patients but this concept was still not yet be demonstrated in hemodialysis patients (Bammens et al., 2005; Pham et al., 2008). In this study, RRF was lost in most patients undergoing long-term HD. Consequently, uremic toxins may accumulate in the serum in patients with decreased residual renal function. However, our results revealed that the patients who underwent long-term HD had higher serum concentrations of unbound and total IS than those who underwent short-term HD. The same was not true for the serum concentrations of p-cresol.

The serum levels of IS and p-cresol are known to be influenced by dietary habits (Wengle and Hellstrom, 1972); however, whether this correlation is influenced by other factors, or only specific dietary habits remains unclear. A previous study revealed that serum albumin is negatively correlated with the serum *p*-cresol level (De Smet et al., 2003). In our study, no specific correlation was determined for the serum albumin concentration. The bowel habit was a possible source influencing p-cresol and IS absorption and it was changed by age. Based on our study, age was not an independent factor to affect serum p-cresol and IS (Lin et al., 2011). As for the aspect of drugs, one prospective phase I/II study indicated 4 weeks of oligofructose inulin significantly lowered serum p-cresylsulfate concentration in HD patients (Meijers et al., 2010). AST-120, one kind of oral charcoal absorbent, was also reported to have effect of lowering the serum IS level (Fujii et al., 2009). Therefore, clinicians are devoted to study how to lower serum IS and p-cresol levels. Our findings can provide clinicians more references.

In conclusion, our results revealed that the serum levels of IS showed an increasing trend in patients undergoing long-term HD. However, the serum levels of *p*-cresol were not associated with the duration of HD.

ACKNOWLEDGEMENTS

The authors thank all the patients involved in this investigation. This study was supported in part by a Grant from Taiwan National Science Council (NSC 100-2314-B-195-012).

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