ORIGINAL

Organ distribution of endogenous *p*-cresol in hemodialysis patients

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Abstract : Background : *p*-Cresol concentrations are high in the blood of hemodialysis (HD) patients. However, its organ distribution has not yet been investigated in detail. We herein report the distribution of *p*-cresol in HD patients from forensic autopsy cases. Methods : *p*-Cresol was measured in the blood, urine, lungs, liver, and kidneys from 4 HD and 4 non-HD cases. Samples were extracted with *p*-cresol-d8 (internal standard), derivatized, and injected on the GC-MS. Results and Discussion : The total urinary *p*-cresol/Cr was 79.73 ng/ml in HD cases, which was 16-fold higher than that in non-HD cases. *p*-Cresol in the blood and kidneys were 30-fold higher or more at 11.92 and 13.08 μg/mL(g), respectively. *p*-Cresol in the liver and lungs were approximately 20-fold higher at 4.82 and 9.99 μg/g, respectively. *p*-Cresol was markedly increased in not only the blood, but also the urine and organs of HD cases. The distribution of *p*-cresol in the blood, urine, and organs differed between HD and non-HD cases. In HD cases, the percentages of conjugated (C) and protein-bound conjugated (PC) urinary *p*-cresol were 57 and 41%, respectively. C and PC *p*-cresol was 66% and 25% in the kidneys, respectively, and similar results were obtained in the lungs. J. Med. Invest. 66 : 81-85, February, 2019

Keywords **:** *p-cresol, hemodialysis patients, chronic kidney disease, organ distribution, forensic autopsy case*

INTRODUCTION

In the absence of external exposure, *p*-cresol (4-methylphenol ; a volatile phenol, one of the uremic toxins, with a molecular weight of 108.1 Da) is a product of the metabolism of tyrosine by intestinal anaerobic bacteria (1-4). During passage through the colonic mucosa and liver, *p*-cresol is conjugated to *p*-cresylsulfate and *p*-cresylglucuronide (5-8), which are then excreted in the urine, and kidney dysfunctions result in the accumulation of uremic toxins in the circulation and tissues.

Phenols, including *p*-cresol, are known to accumulate in uremic serum (9-14). Previous studies suggested that the presence of *p*-cresol is related to overall mortality (15), cardiovascular diseases (16), infectious diseases (17), and uremic symptoms (18) in hemodialysis (HD) patients.

Pioneering research has focused on the concentration and toxicity of *p*-cresol. *p*-Cresol has been shown to principally circulate in the form of its sulfate conjugate (8, 19). It binds tightly to plasma proteins, mostly albumin (molecular weight : 66 kDa). Therefore, this toxin is incompletely removed by HD even though it has a sufficiently low molecular mass to pass through the dialysis membrane (15, 19). However, few studies have investigated the organ distribution of *p*-cresol in HD patients.

In forensic autopsy cases, a toxicological analysis contributes important information for diagnosing the cause of death. In the routine toxicological screening of blood, we encounter high *p*-cresol concentrations in HD cases. Thus, we investigated the blood, urine, and organ distribution of *p*-cresol in forensic autopsy cases of HD patients.

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In the present study, only a small number of cases were examined because difficulties were associated with identifying appropriate cases in forensic autopsies. However, the results obtained are novel.

MATERIALS AND METHODS

Cases

A total of 110 cases were autopsied within 48 hours of the postmortem interval (PMI) between April 2015 and October 2017 in the Department of Forensic Medicine of Fukuoka University. Among the 110 cases, only 4 were HD patients. From the remaining 106 cases, 4 cases without any lesions or injuries on the target organs were chosen as "non-HD" cases. All specimens, including blood, urine, lung, liver, and kidney, were maintained at -30°C until used.

Table 1 Summary of examined cases

Cases	Age	Sex	Cause of death	Kidney[a] (g)	HD duration
HD-1	60s	M	Drowning	187	< 0.5 year
HD-2	80s	F	Bleeding from the shunt site	75	> 10 years
HD-3	80s	M	Pesticide poisoning	205	< 0.5 year
HD-4	60s	M	Carbon monoxide poisoning	133	unknown
non-HD-1	50s	F	Cardiac tamponade by stab wound	256	
non-HD-2	50s	F	Traumatic brain injury	246	
non-HD-3	40s	M	Hypothermia	397	
non-HD-4	30s	M	Drowning	284	

[a]Kidney : total weight of both kidneys
Abbreviations : HD, hemodialysis

A summary of HD and non-HD cases is shown in Table 1. Kidney weights were markedly lower in HD cases than in non-HD cases.

Materials

Standards of *o*-, *m*-, and *p*-cresol were purchased from Wako Pure Chemical Industries (Tokyo, Japan). The deuterated internal standard (IS), *p*-cresol-d8, was obtained from C/D/N Isotopes Inc. (Quebec, Canada). NaCl, $MgSO_4$, ethyl acetate, acetic anhydride, and acetyl chloride were also obtained from Wako. BSTFA + TMCS (99 : 1, v/v, 100 µL) were purchased from Supelco (Bellefonte, USA). n-Propyl acetate, methanol, pyridine, and heptane were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Concentrated sulfuric acid was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The standard of creatinine was purchased from Katayama Chemical, Inc. (Osaka, Japan). The deuterated internal standard (IS), creatinine-d3, was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Amicon® Ultra - 4 centrifugal filter units were purchased from Merck KGaA (Darmstadt, Germany).

Analysis of p-cresol

Preparation of case samples

Blood, urine, and organ samples were prepared according to the following methods. A 0.1-mL aliquot of sample blood and 0.9 mL of distilled water were mixed. A 0.01-mL aliquot of sample urine and 0.99 mL of distilled water were mixed. A 0.1-g sample of organ tissue was homogenized in a bead crusher with 1 mL of distilled water.

Pretreatment before extraction

An outline of the procedure used is showed in Figure 1. "Free *p*-cresol" is non-protein-bound and unconjugated *p*-cresol (F *p*-cresol). "Conjugated *p*-cresol" is non-protein-binding conjugated *p*-cresol (C *p*-cresol). "Total *p*-cresol" includes free *p*-cresol, protein-bound conjugated *p*-cresol, and conjugated *p*-cresol.

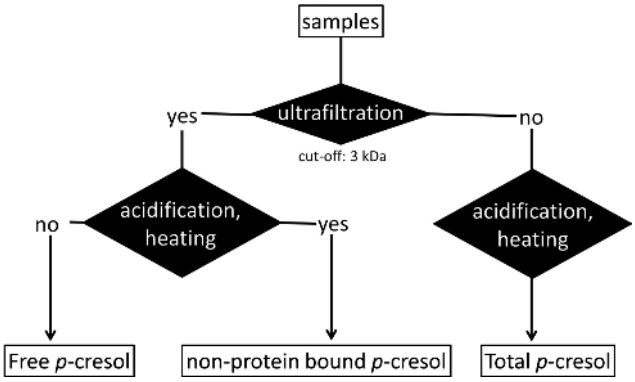

Fig. 1 Flow chart of *p*-cresol extraction and fractionation
Figure 1 shows *p*-cresol extraction in the present study by a flow chart. The sample is separated with a filter with a molecular weight of 3 kDa. When the filtrate is analyzed, free *p*-cresol is obtained. When the filtrate is treated by heat and acid, non-protein bound *p*-cresol is analyzed. Total *p*-cresol is obtained when the original sample is treated by heat and acid without filtering.

F p-cresol

The solution was added to the centrifugal filter unit. After centrifugation at 3500 rpm for 60 min, all of the blood and urine filtrates were added to a glass test tube. Regarding tissues, 0.5 mL of the filtrate and 0.5 mL of distilled water were added to the glass test tube.

F and C p-cresol

The solution was added to the centrifugal filter unit. After centrifugation at 3500 rpm for 60 min, all of the blood and urine filtrates with 0.1 mL of concentrated sulfuric acid were added to a glass test tube. Regarding tissues, 0.5 mL of the filtrate, 0.5 mL of distilled water, and 0.1 mL of concentrated sulfuric acid were added to the glass test tube. The tube was immediately sealed, and after thorough mixing, was incubated at 90°C for 30 min. After heating, the solution was allowed to cool to room temperature.

Total p-cresol

The solution and 0.1 mL of concentrated sulfuric acid were added to a glass test tube. Regarding organ samples, 0.5 mL of distilled water, 0.1 mL of concentrated sulfuric acid, and 0.5 mL of the homogenized sample was added to a glass test tube. The tube was immediately sealed, and after thorough mixing, was incubated at 90°C for 30 min. After heating, the solution was allowed to cool to room temperature.

Extraction

Two milliliters of ethyl acetate, 1 mL of n-propyl acetate, IS solution (*p*-cresol-d8, 1 µg), and 0.5 g NaCl were added to the sample, followed by thorough mixing. After centrifugation at 3000 rpm for 5 min, the organic layer was transferred into another glass test tube and 0.2 g MgSO4 and 0.1 g NaCl were added. After thorough mixing and centrifugation at 3000 rpm for 1 min, the organic layer was transferred to another glass test tube, and evaporated to just short of dryness under nitrogen. The sample was heated using a microwave at 500 W for 30 sec, and 100 µl of BSTFA + TMCS (99 : 1, v/v) was immediately added to the tube. The test tube was sealed and heated using a microwave at 500 W for 90 sec twice (microwave-accelerated derivatization of *p*-cresol). The solution was placed in an autosampler vial for a GC-MS analysis.

Extraction of creatinine

A 0.01-mL aliquot of sample urine, 2 mL of methanol, and IS solution (creatinine-d3, 10 µg) were added to a glass test tube. After mixing, the solution was evaporated to dryness under nitrogen. A total of 0.1 mL of ethyl acetate, 0.01 mL of pyridine, 0.05 mL of acetic anhydride, and 0.01 mL of acetyl chloride were added in this order to the sample, followed by thorough mixing. The test tube was sealed and heated using a microwave at 500 W for 30 sec six times (microwave-accelerated derivatization of creatinine). A total of 0.05 mL of heptane was added to the sample. After mixing and centrifugation, the supernatant was placed in a vial for a GC-MS analysis.

Chromatography conditions

The GC-EI-MS/MS system was GCMS-TQ8030 (Shimadzu, Kyoto, Japan). The column used was ZB SemiVolatiles (2 m × 0.18 mm i.d., film thickness of 0.5 µm, Phenomenex, USA) / BPX5 (4 m × 0.15 mm i.d., film thickness of 0.25 µm, SGE Analytical Science, Australia).

Electron ionization was employed at a voltage of 70 eV. The carrier gas was helium delivered at a constant flow of 2.9 mL/min. The oven temperature was initially 40°C for 0.5 min, increased to 200°C at 70°C/min, increased to 290°C at 50°C/min, and held for 4 min. The injection, interface, and ion source temperatures were all set to 280°C. The sample was injected in the split mode (1 : 10).

Regarding *p*-cresol, Multiple Reaction Monitoring (MRM) transitions and collision energies were assessed through injections of individual standards. The precursor ions, product ions, and collision energies for *p*-cresol-TMS and *p*-cresol-d8-TMS were *m/z* 165.0 to 91.1 (9V) and *m/z* 172.0 to 98.2 (9V), respectively.

The concentration of creatinine was quantified in the SIM mode. The selected ions for creatinine-2AC (acetyl) and creatinine-d3-2 Ac were *m/z* 113.0 and *m/z* 158.0, respectively.

Quantitative analysis

Calibration curves were constructed by plotting the peak area ratios of *p*-cresol to *p*-cresol-d8 (1 μg). Regarding blood and urine samples, the internal standard method was employed. A calibration curve was constructed after the analysis of control blood/urine spiked with 5 known amounts of *p*-cresol. Regarding organ samples, the standard addition method for calibration was employed. In order to make a calibration curve for each organ, 4 amounts of *p*-cresol and *p*-cresol-d8 at a constant concentration were spiked into 0.1-g tissue samples.

The concentration of creatinine was calculated from the peak area ratios of creatinine to creatinine-d3 (10 μg).

Ethics

This study obtained and executed approval by the Ethics Committee on Medical Studies in Fukuoka University (2017M007).

RESULTS

The concentrations of total *p*-cresol and its 3 fractions : F, C, and protein-binding conjugated (PC) *p*-cresol, were summarized in Table 2. The urinary *p*-cresol concentration was corrected by the urinary creatinine (Cr) concentration.

Only a small total *p*-cresol concentration was detected in non-HD cases. In HD cases, total *p*-cresol concentrations in the blood, lungs, liver, kidneys, and urine were 11.92, 9.99, 4.82, 13.08 μg/mL or μg/g, and 79.73 ng/mL, respectively. Blood *p*-cresol concentrations were 30-fold higher than those in non-HD cases. Urinary *p*-cresol/Cr concentrations were also markedly higher in HD cases than in non-HD cases (4.79 ng/mL). *p*-Cresol concentrations in the organs were also significantly higher in HD cases than in non-HD cases ; *p*-cresol concentrations were 20-fold higher in the lungs and liver and 40-fold higher in the kidneys.

Table 2 Distribution of *p*-cresol in blood, urine, and organs

Sample	*p*-Cresol concentrations			
	Free	Conjugated	Protein binding-conjugated	Total
Blood	0.24±0.11 (0.04±0.01)	4.22±1.15 (0.03±0.01)	7.46±0.95 (0.31±0.08)	11.92±2.12 (0.38±0.07)
Lung	0.37±0.08 (0.10±0.01)	6.39±0.78 (0.13±0.06)	3.23±1.19 (0.22±0.08)	9.99±1.96 (0.45±0.12)
Liver	2.59±0.54 (0.11±0.01)	0.77±0.29 (0.06±0.03)	1.46±0.28 (0.08±0.05)	4.82±0.77 (0.26±0.07)
Kidney	1.16±0.46 (0.15±0.02)	8.64±2.58 (0.04±0.02)	3.27±1.37 (0.16±0.09)	13.08±3.60 (0.35±0.11)
Urine[a]	1.58±0.72 (0.38±0.08)	45.22±10.01 (2.83±1.42)	32.93±9.26 (1.59±0.54)	79.73±18.64 (4.79±1.68)

The reference value of non-HD cases is described in the parentheses.
[a]*p*-cresol concentration/Cr
Unit ; μg/ml for blood ; μg/g for organs, and ng/ml for urine
Data are shown as means± standard error.

p-Cresol concentrations and percentages of F, PC, and C *p*-cresol were shown in pie charts (Fig. 2). In HD cases, the percentages of C and PC urinary *p*-cresol were 57 and 41%, respectively. C *p*-cresol was 66% and PC *p*-cresol was 25% in the kidneys. In blood, *p*-cresol was mainly present as PC *p*-cresol (63%). In the liver, F *p*-cresol was the main component of *p*-cresol (54%). The composition of *p*-cresol in the lungs was very similar to that in the kidneys.

DISCUSSION

In the present study, we investigated an analytical method for *p*-cresol not only in blood and urine, but also in organs. We also attempted to reveal the organ distribution of *p*-cresol and its metabolites.

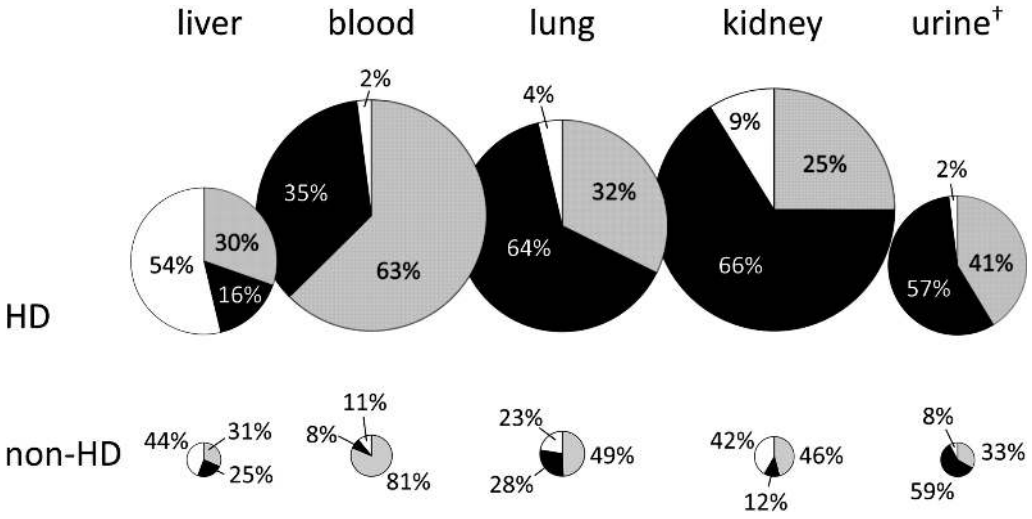

Fig. 2 Pie chart of the distribution of *p*-cresol in blood, urine, and organs
□ Free *p*-cresol
■ protein-binding conjugated *p*-cresol
■ conjugated *p*-cresol
† urine : *p*-cresol/Cr
Figure 2 shows the *p*-cresol concentrations in blood, urine, and organs from Table 2. The total concentration is shown by the size of the circle, and the percentage of each concentration of F, PC, and C *p*-cresol is expressed in the pie chart.

Measurement of p-cresol fractions : F, C, and PC p-cresol

In the human body, *p*-cresol is synthesized in the large intestine by intestinal bacteria (1-4). It is then absorbed by the intestinal wall, conjugated, and transported in the blood via the portal vein. (5-8). Following intestinal absorption, it is also conjugated and metabolized in the liver after general circulation and excreted in the urine. Recent studies reported a close relationship between the kidneys and gastrointestinal (GI) tract-frequently referred to as the kidney-gut axis-in patients with chronic kidney disease (CKD). Numerous molecules, which are either excreted or metabolized by the kidneys, accumulate in patients with CKD (20-23).

p-Cresol exists in various forms in blood, urine, and organs, namely, F, C, and PC *p*-cresol. We attempted to analyze the concentrations of *p*-cresol and its metabolites in the organs of HD patients using heat and acid treatments and GC-MS (8, 15).

p-Cresol concentrations in uremic serum have been assessed using colorimetric nitroaniline diazotization (14), HPLC (7, 24), gas chromatography (GC) (13), and gas chromatography/mass spectrometry (GC/MS) (8, 15). We analyzed *p*-cresol concentrations using the method described by H. de Loor *et al.* (8). In their study, most *p*-cresol was in its sulfated form, more than 95%, and a small proportion was glucuronidated, less than 5%, in serum. They described also that this method, acid and heat deproteinization, can convert almost all of *p*-cresylsulfate into *p*-cresol present in the serum. We analyzed F, C, and PC *p*-cresol individually by their method.

Distribution of p-cresol in blood, urine, and organs

Total blood *p*-cresol concentrations were approximately 30-fold higher in HD cases than in non-HD cases. This result was consistent with previous findings showing that the blood concentrations of cresol and these sulfates were significantly higher in HD patients than non-HD individuals (8, 13, 14). Total *p*-cresol concentrations in urine, corrected by the urinary Cr of HD cases, were also approximately 16-fold higher than in non-HD cases. The liver, kidneys, and lungs were examined as target organs. *p*-Cresol concentrations in the kidneys were approximately 40-fold higher in HD cases than in non-HD cases. *p*-Cresol concentrations were also significantly higher, by approximately 20-fold, in the liver. Therefore, *p*-cresol appears to accumulate in the organs of HD patients because of compromised excretion from the kidneys. Two of the 4 HD cases had a HD duration of less than 6 months (Table 1). These 2 patients had a higher concentration of blood *p*-cresol than the others (Table 3). In both of these cases, the deceased did not like going to the doctor, so treatment was not started until there was a risk for death. Therefore, the blood *p*-cresol concentration in these cases might reflect the stage of CKD (25) rather than the duration of HD.

Percentages of F, C, and PC p-cresol in blood, urine, and organs

In non-HD cases, the percentage of each *p*-cresol type in the blood was as follows : 81% as PC, 8% as C, and 11% as F *p*-cresol. This composition was markedly different in the liver : F *p*-cresol was 44%, PC *p*-cresol was 31%, and C *p*-cresol was 25%. F *p*-cresol is absorbed, partly conjugated to C *p*-cresol in the intestinal wall, and is transported to the liver by the portal vein (5-8). Therefore, the percentage of F *p*-cresol is higher than in the blood. F and PC *p*-cresol are the main forms in the kidneys and liver, which differs from that in the blood. In urine, *p*-cresol was excreted as 59% of C, 33% of PC, and 8% of F *p*-cresol. Therefore, metabolized *p*-cresol was mainly excreted. The percentages of PC *p*-cresol in the lungs and kidneys were similar at 49 and 46%, respectively, whereas those of C *p*-cresol markedly differed at 28 and 12%, respectively. Since C *p*-cresol is excreted in the urine, the percentage of C *p*-cresol may be lower in the kidneys than in the lungs.

We investigated the percentages of F, C, and PC *p*-cresol in HD and non-HD cases. In the urine, no significant differences were observed between HD and non-HD cases, and the percentages of C and PC *p*-cresol in HD cases were 57 and 41%, respectively. *p*-Cresol is excreted into urine as conjugated *p*-cresol ; however, the volume of urine generated by HD patients is decreased. While the composition of conjugated *p*-cresol in the urine did not significantly change, conjugated *p*-cresol accumulated in the body, and was distributed in various organs. Thus, the composition of *p*-cresol in HD cases markedly differed from non-HD cases, except in urine : for example, the percentages of C and PC *p*-cresol in the kidneys of HD cases were 66 and 25%, respectively. This high percentage of C *p*-cresol in various organs was a significant difference between HD and non-HD cases. The percentage of PC *p*-cresol in blood, 63%, was markedly higher than that in other organs and urine. Accumulated C *p*-cresol may stably bind to proteins in blood. The composition of *p*-cresol in the lungs was very similar to that in the kidneys. Therefore, the lungs reflected the composition of *p*-cresol in the kidneys. In the liver, the composition of *p*-cresol was similar between HD and non-HD cases. In HD patients, *p*-cresol also accumulated in the liver, but the normal functioning liver might conjugate and bind *p*-cresol almost at the same ratio in non-HD patients. C *p*-cresol concentrations have been shown to positively correlate not only with CKD, but also cardiovascular disease (CVD) mortality (25). We investigated C *p*-cresol concentrations in HD cases. C *p*-cresol accounted for only 16% of total *p*-cresol in the liver with much higher percentages in the blood, urine, and other organs, especially as high as 65% in the lungs and kidneys. High C *p*-cresol concentrations in HD patients may induce CVD. Free *p*-cresyl sulfate is associated with vascular calcification and is a predictor of overall and CVD mortality in CKD patients (25). In the present study, we also confirmed higher C *p*-cresol concentrations in HD cases than in non-HD cases, and that vascular damage was present in all HD cases. High-grade arteriosclerosis was observed in the thoraco-abdominal aorta of all HD cases. All HD cases showed sclerosis or stenosis of the coronary and basilar arteries. Two of the 4 HD cases also had a history of myocardial infarction (MI) and coronary artery bypass grafting (CABG) (Table 3). The results of the present study confirm previous studies, that a high concentration of C *p*-cresol might be related to CVD in HD cases.

The removal of *p*-cresol from the body is important for reducing CKD and CVD mortality (25). *p*-Cresol cannot be removed by HD because it binds to proteins in the blood (24). The present results revealed that 60% of *p*-cresol existed as PC *p*-cresol in the blood. Heat and acid treatments are needed in order to separate the PC and C *p*-cresol forms. Therefore, difficulties are associated with removing *p*-cresol that accumulates in the blood and organs from the body. Another strategy is to control the production and absorption of *p*-cresol in the intestines.

CONCLUSION

p-Cresol concentrations are high in the blood of HD patients. However, its distribution in the body has not yet been investigated in detail. We herein reported the distribution of *p*-cresol in HD patients.

In the present study, *p*-cresol concentrations were markedly increased not only in the blood, but also the urine and organs of HD cases. The percentages of C and PC urinary *p*-cresol were 57 and 41%, respectively. C *p*-cresol was 66% and PC was 25% in the kidneys. The composition of *p*-cresol in the lungs was very similar to that in the kidneys. The composition of *p*-cresol, namely, F, C, and PC *p*-cresol, markedly differed between HD and non-HD cases. A high concentration of *p*-cresol and high percentage of C *p*-cresol

Table 3 Blood Concentration of *p*-cresol and cardiovascular damage in HD cases

	Blood *p*-Cresol concentrations				Cardiovascular damage			
Cases	Free	Conjugated	Protein binding-conjugated	Total	AS	CS	BS	MI/CABG
HD-1	0.59	7.75	10.46	18.80	+++	++	+	−
HD-2	0.08	3.36	5.21	8.65	+++	+++	+++	+
HD-3	0.22	4.34	7.45	12.01	+++	+++	+++	+
HD-4	0.07	1.41	6.74	8.22	+++	+++	++	−

Abbreviations : HD, hemodialysis ; AS, arteriosclerosis in the thoraco-abdominal aorta ; CS, arteriosclerosis or stenosis in the coronary artery ; BS, arteriosclerosis in the basilar artery ; MI, myocardial infarction ; CABG, coronary artery bypass grafting
Unit : μg/ml for concentration
AS and BS, + mild ;++ medium ;+++ severe
CS, + sclerosis ; ++ stenosis < 50% ; +++ stenosis > 50%

may induce CVD.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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