Original Article

Role of Gut-derived Uremic Toxins on Oxidative Stress and Inflammation in Patients with Chronic Kidney Disease

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Abstract

Several cardiovascular disease (CVD) risk factors have been identified among patients with chronic kidney disease (CKD). Gut-derived uremic toxins (GDUT) are important modifiable contributors in this respect. There are very few Indian studies on GDUT changes in CKD. One hundred and twenty patients older than 18 years diagnosed with CKD were enrolled along with forty healthy subjects. The patients were classified into three groups of forty patients based on stage of CKD. Indoxyl sulfate (IS), para cresyl sulfate (p-CS), indole acetic acid (IAA), and phenol were estimated along with the assessment of oxidative stress (OS), inflammatory state, and bone mineral disturbance. All the GDUT increased across the three groups of CKD. All patients had higher levels of malondialdehyde (MDA), ferric reducing ability of plasma (FRAP), high-sensitivity C-reactive protein (hsCRP), and interleukin-6 (IL-6) as compared to controls. IS and IAA showed positive association with MDA/FRAP corrected for uric acid, whereas IS and p-CS showed positive association with IL-6. IS, IAA, and phenol showed a positive association with calcium × phosphorus product. GDUT increase OS and inflammatory state in CKD and may contribute to CVD risk.

Keywords: *Cardiovascular disease risk, chronic kidney disease, gut-derived uremic toxins*

Introduction

Several cardiovascular disease (CVD) risk factors have been identified among patients with chronic kidney disease (CKD).[1] The presence of CKD itself has been identified as a CVD risk factor,[2] contributing to endothelial dysfunction. One feature responsible for this is accumulation of uremic toxins. Intestine is an important source of some uremic toxins. Apart from acting as a route for entry of uremic toxins, intestine undergoes changes in the composition of fermenting flora, leading to altered processing of uremic toxin precursors.[3] The gut-derived uremic toxins (GDUT) include endogenously produced molecules without interference by intestinal absorption (uric acid, xanthine), ingested exogenous toxins (advanced glycation end-products), and ingested exogenous products undergoing metabolic modification (derivatives of amino acids, phenol, and indoles). GDUT release inflammatory substances by leukocytes causing endothelial dysfunction.[4] Studying GDUT levels have come to focus with the development of strategies to modify intestinal absorption and metabolism of these toxins with the use of pre/probiotics, dietary modification, and adsorbent therapies. Available literature on GDUT is mostly from the western countries. The GDUT levels can also depend on serum albumin, ethnic variations, and dietary habits,[5] thereby indicating the need for Indian data. However, there are very few Indian studies.[6] Hence, the present study was taken up to estimate GDUT, namely, indoxyl sulfate (IS), para cresyl sulfate (p-CS), indole acetic acid (IAA), and phenol along with the role of GDUT on oxidative stress (OS), inflammation, and mineral metabolism in various stages of CKD in a South Indian cohort.

Material and Methods

The present study was conducted between September 2011 and December 2013 in the Department of Biochemistry, Sri Venkateswara Institute of Medical Sciences, Tirupati, Andhra Pradesh, India. One hundred and twenty patients older than 18 years of age diagnosed to have CKD and willing to participate were enrolled along with forty healthy subjects. The study was approved by the Institutional Ethics

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Committee. The patients were classified into three groups of forty patients each (G1 = stages 1 and 2 or early CKD; G2 = stages 3 and 4 or advanced CKD; G3 = end-stage renal disease) based on estimated glomerular filtration rate (eGFR) calculated using Cockcroft-Gault formula.[7] Patients undergoing hemodialysis/peritoneal dialysis, those on antioxidant therapy or vitamin supplementation, taking pro/prebiotics, anti-inflammatory/immunosuppressive drugs, and patients with active inflammatory disease or with abnormal liver function were excluded from the study.

Five milliliters of the peripheral venous blood was collected into heparinized bulb. Plasma was separated and was either analyzed immediately or stored at −80°C. GDUT were estimated using high-performance liquid chromatography with a fluorescence detector[8] using Schimadzu HPLC system (LC-20A, Kyoto, Japan). Serum deproteinization and bound uremic solutes displacement were done as part of sample pretreatment. Mobile phase A consisted of 2.76 g/L (20 mM) NaH_2PO_4, H_2O, and 1.85 g/L (5 mM) tetrabutylammonium iodide in water, and mobile phase B was acetonitrile. For elution, a 1.5 mL/min isocratic flow with 22:78 of A: B mobile phase was used. Twenty microliters of the sample was spiked, and quantification was done using fluorescence detection at specific excitation and emission wavelengths for individual molecules. The concentrations of uremic solutes were calculated using the standard calibration curves using fluorescence detector. The retention times were 4.8 min for phenol, 5.9 min for IAA, 12.5 min for IS, and 16.5 min for p-CS. Three samples spiked to study recovery showed 93% recovery in the normal range and 97% in the uremic range.

To study the effect of OS, malondialdehyde (MDA) and ferric reducing ability of plasma (FRAP) were measured using spectrophotometric methods. The effect of inflammation was assessed by the measurement of high-sensitivity C-reactive protein (hsCRP) and interleukin-6 (IL-6). Nitric oxide (NO) levels were estimated to study the status of endothelial function. Calcium (Ca) and phosphorus (P) were estimated for assessing the mineral metabolism status. Urea and creatinine were estimated for assessing renal function. MDA, FRAP, and NO were estimated using spectrophotometric methods.[9-11] Urea, creatinine, Ca, P, and hsCRP were quantified using commercially available kits on Beckman Synchron autoanalyzer (Beckman Coulter, USA). IL-6 was estimated by ELISA using commercial kits on ChemWell analyzer (Awareness Technology, USA). A comprehensive score was calculated to have an estimate of the sum effect of GDUT on the pathophysiological factors for CVD risk studied. MDA/FRAP corrected for uric acid (FRAP_U), IL-6, and Ca × P product were included in the generation of the score. Values below or equal to control median level of each of the parameters were taken as no risk (score of 0), up to 25% above control median were given a score of 1, between 25% and 50% above control median were given a score of 2, and more than 50% above control median were given a score of 3.

Statistical analysis

Kruskal–Wallis test and Mann–Whitney U-test were used to test the significance of difference in parameters studied. Data for uremic toxins were normalized by logarithmic transformation for regression analysis to study association between uremic toxins and CVD risk factors. A $P < 0.05$ was considered statistically significant. Analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Demographic data and the biochemical parameters studied are presented in Table 1. The scatterplots showing the distribution of GDUT are shown in Figure 1. There was an increase in all the GDUT across the three groups of CKD. Plasma levels of IS and p-CS were increasing with progression of CKD. The increase found in IS, IAA, and phenol was earlier to p-CS. All patients with CKD had significantly higher levels of MDA, FRAP, hsCRP, IL-6, and calculated comprehensive score ($P < 0.001$) compared to controls. NO levels showed a significant decrease ($P < 0.001$) compared to controls.

The association of GDUT with renal function and CVD risk factors is shown in Table 2. All the four uremic toxins showed significant negative association with eGFR. IS and IAA showed significant positive association with MDA and MDA/FRAP_U. IS and p-CS showed significant positive association with IL-6. IS, IAA, and phenol showed a significant positive association with Ca × P product. Only IS showed a positive association with the comprehensive score, indicating its involvement in all the three pathophysiological factors studied.

Discussion

Uremic toxins have been identified to be important links in the causation of accelerated atherosclerosis in patients with CKD.[12] GDUT have come to particular focus with the development of strategies to reduce intestinal absorption of these toxins. In the present study, all the GDUT studied, i.e., IS, p-CS, IAA, and phenol, were found to be higher in CKD patients ($P = 0.009$ for IS, $P < 0.001$ for p-CS and phenol, $P = 0.001$ for IAA) compared to controls. The levels of these toxins also increased progressively with increasing stage of CKD (IS: $P < 0.001$; p-CS: $P = 0.003$; IAA: $P = 0.001$; phenol: $P = 0.004$). However, except for IS which showed a continuous increase, other GDUT studied did not show further increase after Group 2 CKD [Figure 1]. Similar increase was reported for IS,[13,14] p-CS,[14,15] IAA,[16] and phenol.[17] The mechanisms proposed for increased generation of GDUT include decreased absorption of amino acids resulting in availability of substrate for metabolism by microbes, alteration of gut flora in favor of proteolytic

Table 1: Baseline characteristics and biochemical markers studied in controls and chronic kidney disease patients

Parameters	Controls	Group 1	Group 2	Group 3	Significance
n	40	40	40	40	
Male/female	24/16	26/14	30/10	30/10	
Age (years)	42.15±8.77	53.33±14.94	53.13±7.67	50.78±12.94	ANOVA (G1-G3) *P*=0.586
BMI (kg/m^2)	23.37±3.66	26.71±5.47	23.82±4.74	21.68±3.92	ANOVA (G1-G3) *P*<0.001
Urea (mmol/L)	3.16±0.68	4.85±2.02	9.23±3.13	18.98±6.35	ANOVA (G1-G3) *P*<0.001
Creatinine (µmol/L)	58.41±13.33	82.03±32.12	211.54±73.72	584.15±331.19	ANOVA (G1-G3) *P*<0.001
eGFR (mL/min)	120.91±36.05	87.71±38.73	30.71±11.66	11.96±6.91	ANOVA (G1-G3) *P*<0.001
IS (µmol/L)	16.84±10.91	14.03±14.23	23.28±16.85	97.63±62.93	C versus G3 *P*<0.001; ANOVA (G1-G3) *P*<0.001; G1 versus G2 *P*=0.001; G1 versus G3 *P*<0.001; G2 versus G3 *P*<0.001
p-CS (µmol/L)	6.27±7.20	22.43±35.19	69.49±160.12	132.00±185.67	C versus G1 *P*<0.001; C versus G2 *P*=0.001; C versus G3 *P*<0.001; ANOVA (G1-G3) *P*=0.003; G1 versus G3 *P*=0.001; G2 versus G3 *P*=0.011
IAA (µmol/L)	15.37±11.81	17.79±16.10	30.37±16.54	27.39±15.74	C versus G2 *P*<0.001; C versus G3 *P*<0.001; ANOVA (G1-G3) *P*=0.001; G1 versus G2 *P*=0.001; G1 versus G3 *P*=0.004
Phenol (µmol/L)	8.54±4.79	12.35±9.17	16.14±6.68	14.99±5.40	C versus G1 *P*=0.043; C versus G2 *P*<0.001; C versus G3 *P*<0.001; ANOVA (G1-G3) *P*=0.004; G1 versus G2 *P*=0.003; G1 versus G3 *P*=0.006
MDA (µmol/L)	1.83±0.52	3.27±0.70	4.55±1.89	4.83±2.05	C versus CKD group *P*<0.001
FRAP (mmol/L)	0.84±0.29	1.25±0.16	1.58±0.14	1.56±0.29	C versus CKD group *P*<0.001
MDA/FRAP_U	0.56±0.34	0.94±0.37	1.16±0.57	1.30±0.64	C versus G1 *P*<0.001; C versus G2 *P*<0.001; C versus G3 *P*<0.001; G1 versus G3 *P*=0.029
hs-CRP (mg/L)	1.00±0.65	3.82±3.00	11.05±16.40	4.73±5.11	C versus CKD group *P*<0.001
IL-6 (pg/L)	161.54±223.80	206.35±225.07	268.42±238.70	1412.48±1242.22	C versus CKD group *P*=0.003
Calcium × phosphorus	2.02±0.80	1.71±0.85	2.15±0.90	2.69±0.92	ANOVA (G1-G3) *P*<0.001
NO (µmol/L)	36.18±5.60	28.93±3.28	24.81±4.89	28.16±8.07	C versus CKD group *P*<0.001

Data expressed as mean±SD. G1: Stages 1 and 2 or early CKD, G2: Stages 3 and 4 or advanced CKD, G3: End-stage renal disease, C: Control group, CKD group: All patients with CKD, BMI: Body mass index, eGFR: Estimated glomerular filtration rate, IS: Indoxyl sulfate, p-CS: Para cresol sulfate, IAA: Indole acetic acid, MDA: Malondialdehyde, FRAP_U: Ferric reducing ability of plasma corrected for uric acid, hsCRP: High sensitivity C-reactive protein, IL-6: Interleukin-6, NO: Nitric oxide, CKD: Chronic kidney disease, SD: Standard deviation

microorganisms producing more toxin precursors, abnormal intestinal motility resulting in increased absorption of bacterial toxins along with decreased renal clearance.[18]

Association of gut-derived uremic toxin with renal function

All four GDUT studied except phenol showed significant positive association with creatinine, whereas all showed negative association with eGFR [Table 2]. Meijers *et al*.[19] showed significant positive correlation between total IS and creatinine but not with p-CS, whereas Lin *et al*.[20] observed significant positive correlation with both IS and p-CS. Liabeuf *et al*.[15] and Barreto *et al*.[13] reported significant inverse association of GFR with p-CS and IS, respectively. These and our findings suggest decreased clearance as one of the reasons for accumulation of GDUT.

Gut-derived uremic toxins and oxidative stress

Both OS markers studied, MDA and FRAP were found to be elevated in patients with CKD ($P < 0.001$) as compared to controls. Similar findings were reported in other studies.[21,22] About 60% of the total antioxidant power measured by FRAP assay is contributed by uric acid.[23] Hence, FRAP_U and MDA/FRAP_U were considered as the combined effect of oxygen radical production and nonenzymatic antioxidant defense. MDA/FRAP_U was significantly higher in CKD patients ($P < 0.001$) compared to controls, indicating increased production of oxygen-free radicals as the main reason for OS in CKD. Among the GDUT studied, IS and IAA showed positive association with MDA ($P < 0.001$ and 0.020 for IS and IAA, respectively) as well as with MDA/FRAP_U ($P = 0.011$ and 0.025 for IS and IAA, respectively). These results suggest that the OS in CKD is predominantly due to overproduction of oxygen-free radicals and IS and IAA contribute to the increased production of oxygen radicals.

Rossi *et al*.[24] reported that free IS and p-CS were associated with glutathione peroxidase in patients with stages 3 and 4 CKD. Chao and Chiang[25] reported that uremic toxins are potent inducers of OS and contribute to tissue damage. Studies involving animal models showed that upregulation of cyclo-oxygenase-2 (COX-2), a source of renal reactive oxygen species (ROS) through nicotinamide adenine dinucleotide phosphate (NADPH) dependent or independent pathways, may induce OS.[25,26] IAA was shown to increase

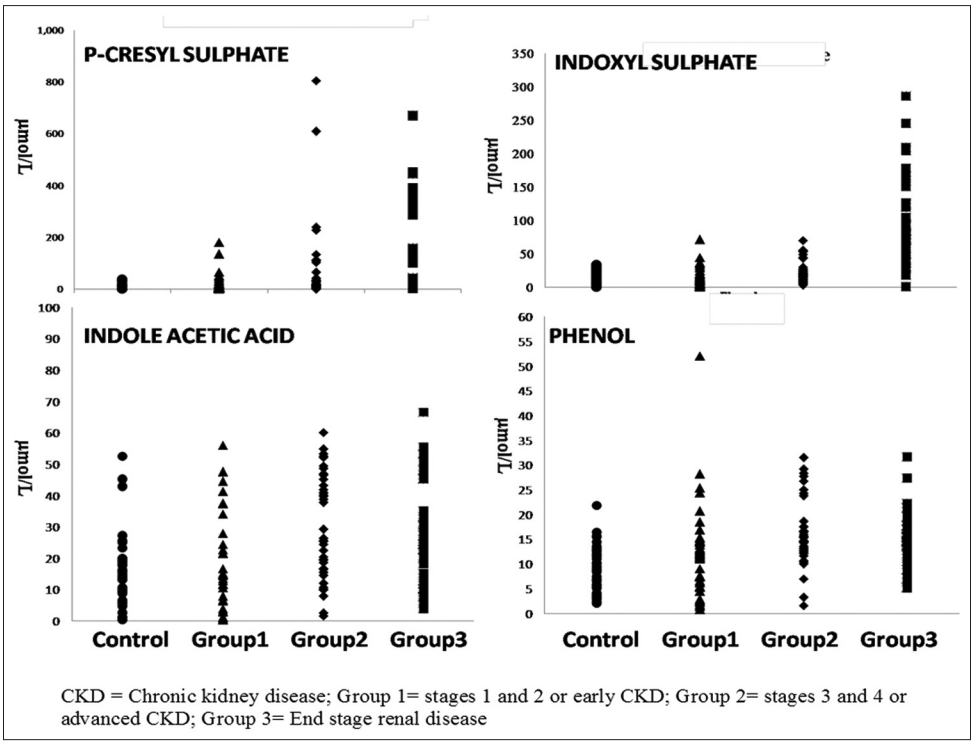

Figure 1: Scatterplots showing the distribution of gut-derived uremic toxins

COX-2 levels, thereby contributing to ROS generation.[16] Dou *et al*.[27] showed that IS significantly increased ROS production in human umbilical vein endothelial cells through activation of NADPH oxidase. They also observed that both oxidized and reduced forms of glutathione were inhibited by IS.

Gut-derived uremic toxins and Inflammation

CKD has been reported to be associated with inflammation. In the present study, both the inflammatory markers studied, hsCRP and IL-6, were elevated in CKD patients when compared with controls ($P < 0.001$). The rise in hsCRP occurred early in CKD and there was no further increase with progression of the disease. However, IL-6 showed a continuous increase with progression of CKD ($P < 0.001$). Among the GDUT studied, IS ($P < 0.001$) and p-CS ($P = 0.044$) showed significant positive association with IL-6. Thus, findings of the present study show that GDUT mainly IS and p-CS have a pro-inflammatory effect.

The increase in the levels of inflammatory markers in CKD patients could be due to their decreased renal clearance and other coexisting factors.[28] Rossi *et al*.[24] found that IS and p-CS correlated significantly with IL-6 in stages 3 and 4 CKD patients. Dou *et al*.[16] found significant positive correlation between IAA and CRP. Accumulated IS is shown to cause injury to tubular cells and induce leukocyte adhesion to endothelial cells through endothelial cell inflammation.[29,30] In experimental models, p-CS promoted expression of inflammatory genes and contributed to renal cell injury.[31] However, some studies found no significant association between uremic toxins and hsCRP.[25] Increased hsCRP levels observed in the present study could be a result of increased synthesis of CRP by liver which is triggered in response to IL-6.[32]

Gut-derived uremic toxins and mineral metabolism

Disturbances in Ca and *P* metabolism are observed in CKD[33] and GDUT have been identified to contribute to this disturbance in mineral metabolism through causing OS in bone and cardiovascular systems.[34] In the present study, there was an increase in Ca × P product with progressing CKD ($P < 0.001$). IS, IAA, and phenol showed significant positive association with Ca × P product ($P = 0.001$, $P = 0.012$, and $P = 0.010$, respectively). A previous study has similarly reported a significant positive correlation of p-CS and IS with Ca × P.[35] IS has been shown to be vasculotoxic by promoting aortic calcification.[36]

To understand the influence of GDUT on the pathophysiological factors leading to increased CVD risk associated with CKD, a comprehensive score was calculated. It was observed that CKD patients had higher risk score when compared to controls (5.47 ± 1.99 vs. 2.85 ± 2.26; $P < 0.001$). IS, which was involved in all the factors, showed positive association with this risk score.

Thus, findings of the present study suggest that GDUT are associated with increased OS, inflammatory state, and bone mineral disturbance. As a result, the increased GDUT levels observed in CKD patients contribute to the increased CVD risk of CKD patients. Hence, treatment in the form of use of pre/probiotics and adsorbent therapies or dietary

Table 2: Association of gut-derived uremic toxins with renal function and cardiovascular disease risk factors

Dependent variable	Predictor	*B*	SE	95% CI for *B*		*P*
				Lower bound	Upper bound	
Creatinine	IS	260.405	39.718	181.738	339.072	<0.001
	p-CS	86.023	34.975	16.757	155.289	0.015
	IAA	114.036	55.056	4.991	223.080	0.041
	Phenol	147.498	89.928	−30.632	325.629	0.104
eGFR	IS	−33.569	6.282	−46.026	−21.111	<0.001
	p-CS	−14.776	4.823	−24.339	−5.214	0.003
	IAA	−37.251	7.491	−52.106	−22.396	<0.001
	Phenol	−46.042	12.394	−70.622	−21.462	<0.001
MDA	IS	0.988	0.273	0.447	1.528	<0.001
	p-CS	0.350	0.224	−0.093	0.794	0.121
	IAA	0.817	0.346	0.132	1.502	0.020
	Phenol	1.043	0.606	−0.157	2.244	0.088
MDA/FRAP_U	IS	0.225	0.087	0.053	0.397	0.011
	p-CS	0.022	0.071	−0.118	0.162	0.754
	IAA	0.241	0.106	0.030	0.452	0.025
	Phenol	0.122	0.189	−0.253	0.498	0.521
IL-6	IS	543.467	135.956	274.190	812.744	<0.001
	p-CS	24.275	11.908	0.667	47.883	0.044
	IAA	84.072	178.518	−269.507	437.650	0.639
	Phenol	202.236	293.924	−379.972	784.444	0.493
Calcium × phosphorus	IS	0.495	0.148	0.202	0.788	0.001
	p-CS	0.131	0.119	−0.106	0.367	0.277
	IAA	0.466	0.183	0.103	0.830	0.012
	Phenol	0.802	0.305	0.198	1.406	0.010
Comprehensive score	IS	1.081	0.304	0.479	1.683	0.001
	p-CS	0.108	0.246	−0.379	0.595	0.661
	IAA	0.574	0.369	−0.157	1.305	0.122
	Phenol	0.598	0.642	−0.674	1.870	0.354

P<0.05 - statistically significant. Comprehensive score based on MDA/FRAP_U, IL-6, and calcium × phosphorus product. *B*: Regression coefficient, SE: Standard error, eGFR: Estimated glomerular filtration rate, IS: Indoxyl sulfate, p-CS: Para cresol sulfate, IAA: Indole acetic acid, MDA: Malondialdehyde, FRAP_U: Ferric reducing ability of plasma corrected for uric acid, IL-6: Interleukin-6, CI: Confidence interval

modifications to reduce the levels of GDUT can be useful in reducing CVD risk in CKD.

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Conflicts of interest

There are no conflicts of interest.

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