8.3 MYCOTOXIN DETECTION IN URINE SAMPLES FROM PATIENTS WITH CHRONIC KIDNEY DISEASE OF UNCERTAIN ETIOLOGY IN SRILANKA

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Mycotoxin Detection in Urine Samples from Patients with Chronic Kidney Disease of Uncertain Etiology in Sri Lanka

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Abstract This was a screening study that aimed to determine the presence of nephrotoxic mycotoxins in urine samples from patients with chronic kidney disease of uncertain etiology in the North Central Province of Sri Lanka. The percentage detection of aßatoxins, ochratoxins and fumonisins in 31 patients were 61.29%, 93.5% and 19.4%, respectively. Geometric means of urinary aßatoxins and ochratoxins were 30.93 creatinine and 34.62 ng/g creatinine in chronic kidney disease of uncertain etiology stage 1Ð2 patients and 84.12 ng/g creatinine and 63.52 ng/g creatinine in unaffected relatives of patients. In chronic kidney disease of uncertain etiology stage 3Ð5 patients, geometric means of urinary aßatoxins and ochratoxins were 10.40 and 17.08 ng/g creatinine, respectively. Non-affected relatives of patients (n=6) had comparable levels of these mycotoxins, but healthy Japanese individuals (n=4) had lower levels than in Sri Lanka. The higher detection rate of urinary ochratoxins in Sri Lankans indicates that exposure is common in the region.

Keywords Chronic kidney disease of uncertain etiology · Sri Lanka · Urine sample · Aßatoxin · Ochratoxin · Fumonisin

High prevalence of chronic kidney disease of uncertain etiology (CKDue) in the North Central Province of Sri Lanka has been reported. The disease predominantly affects male farming communities. Several hypotheses have been made to explain the causal associations between the high prevalence of CKDue in the region and existing environmental factors (Chandrajith et al.2010; Illeperuma et al.2009).

Mycotoxins, such as aßatoxins (AFLs) (Glahn et al. 1994), ochratoxins (OTs) (Sauvant et al. 2005) and fumonisins (FBs) (Badria et al. 1996) are dietary contaminants that are known to possess nephrotoxicity. Animal studies demonstrated ochratoxin (Baudrimont et al.2001; Berndt et al. 1980) and AFB_1 (Grosman et al. 1983) induced proteinurea and decrease of glomerular Þltration rate and tubular reabsorption of glucose. Detection of OT associated with the incidence of endemic nephropathy in other regions has been reported (Castegnaro et al. 2006; Domijan et al.2009). A recent study by Wanigasuriya et al. (2008) has reported that the concentration of OT A in selected food items in the study region was low. Food analysis, in some instances, might not be sufÞcient to establish a relationship with occurrence of diseases due to heterogeneity of toxin distribution over time, and even within a particular food product, casts doubt on the feasibility of sampling plans (Parsons et al.2007. For instance, in some studies (Castegnaro et al.2006), the relationship of

This study is conducted for the Chronic Kidney Disease of Uncertain Etiology Consortium.
Please refer the Appendix section for the full list of members.

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an increase in OTA intake was not found in agreement with the immediate increase of its elimination in urine. In an attempt to overcome this problem and to validate the actual exposure, we screened urinary excretion levels of AFL, OTs and FBs in patients and their relatives living in a CKD endemic community.

Materials and Methods

Ethical approval for this study was obtained from the Ethical Committee of Kyoto University, Japan and the Ethical Review Committees of the Faculty of Medicine, University of Peradeniya, Sri Lanka. The urine samples were originally collected at Medawachchiya and Girandrukotte, Sri Lanka in August 2009 (106 patients and 87 unaffected relatives of CKDue patients) and stored at Ð30°C in the Kyoto University Human Specimen Bank (Koizumi et al.2009). A total of 41 urine samples, 31 from stage 1Ð5 CKDue patients, six from unaffected relatives, and four from healthy Japanese individuals as controls, were randomly selected from each stratum. DeÞnition of CKD and further classiÞcation of the stages were made according to the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines. Patients with a history and current treatment of diabetes mellitus, severe hypertension, urological disease of known etiology, glomerulonephritis, or snake bite were excluded. Creatinine concentration in urine sample was measured by enzyme assay using creatinine amidohydrolase (SRL, Tokyo, Japan).

Urine samples were thawed and centrifuged at 15,000 rpm for 10 min to remove any cellular debris, and the supernatant was used for the determination of mycotoxin level. One milliliter of urine was diluted with 3 mL PBS (pH 7.4). The mixed sample was directly passed through analyte-speciÞc immunoafÞnity columns (R-Biopharm AG, Darmstadt, Germany) at a ßow rate of 1Ð2 drops/s. The column was washed with 20 mL PBS and air was passed through the column for 1 min. The bound mycotoxin was eluted with 3 mL methanol and the eluate was evaporated to dryness using a nitrogen evaporator. The residue was reconstituted with 100µL 10% methanol in water, and analyzed for each mycotoxin with the speciÞc competitive ELISA kits (RIDASCREEN FAST Mycotoxins; R-Biopharm AG) using a microplate spectrophotometer (inÞnite M200 Pro; Tecan, Tokyo, Japan) at 450 nm. ELISA kits for AFL, OTs and FBs recognized aßatoxins B1, B2, G1, G2 and M1; ochratoxins A, B and C, and fumonisins B1, B2 and B3, respectively. External standards of different concentrations and all urine samples were run in duplicate.

Mycotoxin concentration was computed based on the absorbance of Þve standard solutions provided by the immunoassay kit for each mycotoxin. Mean recoveries of AFLs, OTs and FBs from the fortiÞed samples were 79%, 105% and 92%, and the corresponding coefÞcients of variation were 11, 13 and 15, respectively. Detection limits for AFLs, OTs and FBs in the urine samples were 0.005, 0.005 and 0.035 ng/mL, respectively. For values below the detection limit, half of the limit of detection value was assigned. Mycotoxin concentrations are presented in ng/mL and ng/g Cr (creatinine). Statistical signiÞcance of differences between groups was tested by using non-parametric methods (χ^2 test and Wilcoxon two-sample test; $p<0.05$).

Results and Discussion

Study subjects comprised 20 men and 21 women (Table1). The mean (range) age regardless of disease stage (31, stage 1Ð5) was 41.32±15.55 (9Ð65) years, whereas that of unaffected relatives and Japanese controls was 20.67 (6Ð34) years and 45.25 (42Ð53) years, respectively.

Results of urinary AFL, OT and FB levels are shown in Table2. The percentage detection of AFLs, OTs and FBs in patients was 61.29%, 93.5% and 19.4%, respectively. The detection rate of all mycotoxins in stage 1 disease was the highest. Disease stages were classiÞed as early (stage 1 and 2) and late (stage 3Ð5) for examination of concentration differences during disease progression. Detection rates of AFLs in the early and late stages were 78.57% and 47.06%, respectively (χ^2 =9.323; $p<0.001$). OTs were detected in all of the urine samples from 14 patients with early stage disease, whereas the rate of detection at the late stage was 88.24% (n=17) (χ^2 =23.516, $p<0.001$). Both AFLs and OTs were detected in all of the relatives of CKDue patients, but only OTs were detected in the Japanese controls.

The highest AFL concentration in urine samples from CKDue patients was 0.8 ng/mL, whereas 90% of the samples had a concentration <0.044 ng/mL (397.1 ng/g Cr). The 90th percentile for OTs was 0.098 ng/mL (60.85 ng/g Cr). The geometric means of urinary AFLs and OTs were 0.033 ng/mL (30.93 ng/g Cr) and 0.037 ng/mL (34.62 ng/g Cr) in the early stage, and 0.008 ng/mL (10.40 ng/g Cr) and 0.012 ng/mL (17.08 ng/g Cr) in the late stage of the disease. Mean concentration difference for urinary OT level was observed between the early and late stages of the disease (Wilcoxon test,p=0.008). In contrast, comparable concentrations of OTs and AFLs were also observed in the unaffected relatives of CKDue patients ($p>0.05$ compared with all patients). The concentration

Table 1 Baseline characteristics of CKDue patients in Sri Lanka, 2009

Disease stages	Sex Male/female (total)	Age (year) Mean (range)
Stage 1 (slight)	3/4 (7)	24.14 (9D40)
Stage 2 (mild)	6/1 (7)	48.00 (39Đ59)
Stage 1Đ2 (early stage)	9/5 (14)	36.07±15.19 †
Stage 3 (moderate)	3/3 (6)	41.00 (11Đ60)
Stage 4 (severe)	3/3 (6)	47.50 (35Đ58)
Stage 5 (end stage)	3/2 (5)	49.00 (30Đ65)
Stage 3Đ5 (late stage)	9/8 (17)	45.65±14.90 †
Total (CKDue patients)	18/13 (31)	41.32±15.55
Relatives of CKDue patients	2/4 (6)	20.67 (6Đ34)
Japanese controls	0/4 (4)	45.25 (42Đ53)

† Mean±SD

Table 2 Urine concentration of AFL, OT and FB in CKDue patients in Sri Lanka, 2009

Subjects		AFL		OT		FB	
		ng/mL	ng/g Cr	ng/mL	ng/g Cr	ng/mL	μg/g Cr
Stage 1 (n=7)	Range (n > MDL)	NDĐ0.800(6)	NDĐ734.00	0.013Đ0.360 (7)	17.63Đ93.90	NDĐ0.042 (4)	NDĐ0.14
	Mean	0.359	230.21	0.044	39.67	<MĐL	<MDL
	GM	0.092	87.41	0.035	33.33	<MĐL	<MDL
Stage 2 (n=7)	Range (n > MDL)	NDĐ0.037 (5)	NDĐ53.05	0.006Đ0.058 (6)	11.87Đ74.81	NDĐ0.036 (1)	NDĐ0.07
	Mean	0.018	19.58	0.085	65.07	Đ	Đ
	GM	0.012	10.95	0.039	35.95	Đ	Đ
Stage 1Đ2	GM	0.033	30.93	0.037	34.62*	Đ	Đ
Stage 3 (n=6)	Range (n > MDL)	NDĐ0.039 (4)	NDĐ44.74	NDĐ0.028 (5)	8.57Đ41.25	NDĐ0.130 (1)	NDĐ0.19
	Mean	0.023	25.57	0.022	21.76	Đ	Đ
	GM	0.022	18.75	0.016	19.36	Đ	Đ
Stage 4 (n=6)	Range (n > MDL)	NDĐ0.800 (4)	NDĐ991.57	NDĐ0.019 (4)	NDĐ34.27	Đ	Đ
	Mean	0.140	174.82	0.016	18.75	ND	ND
	GM	0.009	12.71	0.012	17.07	Đ	Đ
Stage 5 (n=5)	Range (n > MDL)	ND	ND	0.010 (4)	NDĐ27.06	ND	ND
	Mean	Đ	Đ	0.044	16.56	Đ	Đ
	GM	Đ	Đ	0.080	14.72	Đ	Đ
Stage 3Đ5	GM	0.008	10.40	0.012	17.08*	Đ	Đ
Stage 1Đ5	GM	0.012	17.01	0.020	23.50	Đ	Đ
Relatives controls (n=6)	Range (n > MDL)	0.020Đ0.800 (6)	5.9Đ1000.00	0.032Đ0.223 (6)	28.63Đ278.00	NDĐ0.093 (1)	NDD0.14
	Mean	0.298	249.09	0.104	88.95	Đ	Đ
	GM	0.112	84.12	0.085	63.52	Đ	Đ
Japanese controls (n=4)	Range (n > MDL)	NĐ	NĐ	0.005Đ0.012 (4)	4.4Đ19.40	ND	NĐ
	Mean	Đ	Đ	0.007	9.69	Đ	Đ
	GM	Đ	Đ	0.007	8.14	Đ	Đ

*ND*not detected,*MDL*method detection limit,*GM*geometric mean

* Wilcoxon test for mean OT concentration difference between early and late stages (p=0.008)

Table 3 Urine mycotoxin level in other countries

Mycotoxin type	Detection rate	Mean (range)	Study subjects	Country	References
AFL	61.29%	17.0 (NDÐ991.6) ng/gCr	CKDue patients	Sri Lanka	Present study
	58%	391.0 (19.0Ð19,219.0) pg/g Cr	General population	Czech Republic	(Malir et al.2004)
OT A	100%	37.1 (12.4Ð360.0) pg/mL	CKDue patients (early stage)	Sri Lanka	Present study
	88.24%	12.0 (NDÐ58.2) pg/mL	CKDue patients (late stage)	Sri Lanka	Present study
	100%(n=6)	85.0 (32.0Ð223.0) pg/mL	Relatives of CKDue patients	Sri Lanka	Present study
	61%	13.0 (6.0Ð65.0) pg/mL	Healthy individuals	Hungary	(Fazekas et al.2005)
	43%	7.0 (5.0Ð15.0) pg/mL	Endemic nephropathy	Croatia	(Domijan et al.2009)
	92.20%	22.0 (NDÐ69.0) pg/mL	General population	Portugal	(Duarte et al.2010)
	88%	50.8 (1.0Ð330.0) pg/mL	Endemic nephropathy	Bulgaria	(Castegnaro et al.2006)
	97.6%	191.7 (1.0Ð191.0) pg/mL	Endemic nephropathy	Bulgaria	(Castegnaro et al.2006)
FB	19.4%	(NDÐ130.0 pg/mL)	CKDue patients	Sri Lanka	Present study
	0% (LOD=5 ng/mL)		General population	Portugal	(Silva et al.2010)
	75%	70.1 (NDÐ9312.0) pg/mL	General population	Mexico	(Gong et al.2004)

of OTs in the healthy Japanese individuals was 0.007 ng/mL (8.14 ng/g Cr) which was lower than in the Sri Lankan individuals.

The small sample size of the control subjects and their characteristic differences with the patients limit the comparability of the results. However, the high detection frequency and urinary levels of OTs and AFLs among CKDue patients and their relatives demonstrated the potential human exposure in the region. Findings were also discussed in relation to similar studies in other countries (Table3). The average AFL concentration in urine samples from CKDue patients was markedly higher, by over an order of magnitude, than the level of 0.391 ng/g Cr in the Czech Republic (Malir et al.2004). An FB exposure study in two Portuguese populations has shown no detectable level in urine samples (Silva et al. 2010) and in Mexico 75% detection frequency was observed (Gong et al.2004), whereas minute level of FBs was detected at the early stage of the disease in the present study.

Higher detection of OTs was observed compared with the 61% detection rate among healthy individuals in Hungary and 43% in the endemic nephropathy area in Croatia (Domijan et al.2009), whereas the detection was comparable with the 88Ð97.8% in the endemic nephropathy region of Bulgaria (Castegnaro et al.2006). Although the mean OT level in CKDue patients in our study was higher than the 0.007 ng/mL in Croatia (Domijan et al.2009) and 0.013 ng/mL in Hungary (Fazekas et al. 2005), and was comparable to the 0.022 ng/mL in Portugal (Duarte et al. 2010), the urine concentration levels in half of our CKDue patients were <0.017 ng/mL (n=15). Therefore, the potential sources of exposure to OTs in the region need to be clariÞed.

Animal studies have demonstrated the possibility of higher concentrations of OT A in kidney tissues and low levels in the urine (Zepnik et al. 2003). Likewise, an increase in OT A intake in humans in the region of endemic nephropathy did not result in an immediate increase in its elimination (Castegnaro et al.2006). OT A is characterized by high plasma protein binding potential, therefore, its removal efÞciency might be low (Petzinger and Weidenbach2002; Ringot et al.2006), and it is possible that OT A may accumulate in renal tissue. It is worth noting that the cumulative effect of long-term consumption of products that contain low levels of mycotoxins could contribute to a gradual deterioration of organ function.

This study is believed to be the Þrst to determine the presence of AFLs, OTs and FBs in urine samples from CKDue patients and their relatives living in communities with CKDue. The higher detection rate of OTs in Sri Lanka has led to a working hypothesis that these mycotoxins could be common in the region, which corroborates the need for further exposure assessment, associated with disease occurrence.

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Appendix

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