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

Biruck Desalegn[1], Shanika Nanayakkara[1], Kouji H. Harada[1], Toshiaki Hitomi[1], Rohana Chandrajith[2], Upul Karunaratne[3], Tilak Abeysekera[3], and Akio Koizumi[1] for the Chronic Kidney Disease of Uncertain Etiology Consortium[4]

[1]Department of Health and Environmental Sciences, Kyoto University Graduate School of Medicine, Yoshida, Kyoto 606-8501, Japan

[2]Department of Geology, Faculty of Science, University of Peradeniya, Sri Lanka

[3]Nephrology Unit, Teaching Hospital, Kandy, Sri Lanka

[4]A full list of members is provided in the supplementary note.

Correspondence to: Akio Koizumi M.D., Ph.D.

Department of Health and Environmental Sciences, Graduate School of Medicine, Kyoto University, Yoshida Konoe, Sakyo, Kyoto 606-8501, Japan

Tel: +81-75-753-4456; Fax: +81-75-753-4458

E-mail: Akio.Koizumi@z06.mbox.media.kyoto-u.ac.jp

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 (CKDue) in the North Central Province of Sri Lanka. The percentage detection of aflatoxins (AFLs), ochratoxins (OTs) and fumonisins in 31 patients were 61.29%, 93.5% and 19.4%, respectively. Geometric means of urinary AFLs and OTs were 30.93 ng/g Cr (creatinine) and 34.62 ng/g Cr in CKDue stage 1–2 patients and 84.12 ng/g Cr and 63.52 ng/g Cr in unaffected relatives of patients. In CKDue stage 3–5 patients, geometric means of urinary AFLs and OTs were 10.40 and 17.08 ng/g Cr, 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 OTs in Sri Lankans indicates that exposure is common in the region.

Keywords chronic kidney disease of uncertain etiology, Sri Lanka, urine sample, aflatoxin, 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 the disease in the region and existing environmental factors (Chandrajith et al. 2010; Illeperuma et al. 2009).

Mycotoxins, such as aflatoxins (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. Detection of OT associated with the incidence of endemic nephropathy in some regions has been reported (Castegnaro et al. 2005; 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 sufficient to establish a relationship with occurrence of diseases because heterogeneity of toxin distribution over time, and even within a particular food product, casts doubt on the feasibility of sampling plans (Parson et al. 2007). 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 –30°C in the Kyoto University Human Specimen Bank (Koizumi et al. 2009). A total of 41 urine samples, 31 from stage 1–5 CKDue patients, six from unaffected relatives, and four from healthy Japanese individuals as controls, were randomly selected from each stratum. Definition of CKD and further classification 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-specific immunoaffinity columns (R-Biopharm AG, Darmstadt, Germany) at a flow rate of 1–2 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 100 μL 10% methanol in water, and analyzed for each mycotoxin with the specific competitive ELISA kits (RIDASCREEN FAST Mycotoxins; R-Biopharm AG) using a microplate spectrophotometer (infinite

M200 Pro; Tecan, Tokyo, Japan) at 450 nm. ELISA kits for AFL, OTs and FBs recognized aflatoxins 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.

Mean recovery (CV) of fortified samples was 79% (11) for AFLs, 105% (13) for OTs and 92% (15) for FBs. Detection limits were 0.005 ng/mL, 0.005 ng/mL and 0.035 ng/mL for AFLs, OTs and FBs, 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 significance of differences 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 (Table 1). The mean (range) age regardless of disease stage (31, stage 1–5) was 41.32 ± 15.55 (9–65) years, whereas that of unaffected relatives and Japanese controls was 20.67 (6–34) years and 45.25 (42–53) years, respectively.

Results of urinary AFL, OT and FB levels are shown in Table 2. 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 classified as early (stage 1 and 2) and late (stage 3–5) for examination of concentration differences during disease progression. Detection rates of AFLs in the early and late stages were 78.57% and 47.06%,

106 respectively ($\chi^2 = 9.323$; $P < 0.001$). OTs were detected in all of the urine samples
107 from 14 patients with early stage disease, whereas the rate of detection at the late
108 stage was 88.24% ($n = 17$) ($\chi^2 = 23.516$, $P < 0.001$). Both AFLs and OTs were
109 detected in all of the relatives of CKDue patients, but only OTs were detected in
110 the Japanese controls.

111 The highest AFL concentration in urine samples from CKDue patients was
112 0.8 ng/mL, whereas 90% of the samples had a concentration <0.044 ng/mL (397.1
113 ng/g Cr). The 90th percentile for OTs was 0.098 ng/mL (60.85 ng/g Cr). The
114 geometric means of urinary AFLs and OTs were 0.033 ng/mL (30.93 ng/g Cr) and
115 0.037 ng/mL (34.62 ng/g Cr) in the early stage, and 0.008 ng/mL (10.40 ng/g Cr)
116 and 0.012 ng/mL (17.08 ng/g Cr) in the late stage of the disease. Mean
117 concentration difference for urinary OT level was observed between the early and
118 late stages of the disease (Wilcoxon test, $P = 0.008$). In contrast, comparable
119 concentrations of OTs and AFLs were also observed in the unaffected relatives of
120 CKDue patients ($P > 0.05$ compared with all patients). Healthy Japanese
121 individuals, however, had lower levels of OTs (0.007 ng/mL, 8.14 ng/g Cr) than
122 Sri Lankan individuals had.

123 The small sample size of the control subjects and their characteristic
124 differences with the patients limit the comparability of the results. However, the
125 high detection frequency and urinary levels of OTs and AFLs among CKDue
126 patients and their relatives demonstrated the potential human exposure in the
127 region. Findings were also discussed in relation to similar studies in other
128 countries (Table 3). 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. 2008) and in Mexico 75% detection frequency was observed (Gong et al. 2008), whereas some 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 88–97.8% in the endemic nephropathy region of Bulgaria (Castegnaro et al. 2005). 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. 2004), 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). The potential sources of exposure to OTs in the region need to be clarified.

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. 2005). OT A is characterized by high plasma protein binding potential, therefore, its removal efficiency might be low (Petzinger et al. 2000; Ringot et al. 2006), and it is possible that OT A accumulates 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 first 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 this mycotoxin could be common in the region, which corroborates the need for further exposure assessment, associated with disease occurrence.

Acknowledgments

This work was supported by special coordination funds for promoting science and technology sponsored by the Japan Science and Technology Agency. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have declared that they have no competing interests.

References

Badria FA, Li S, Shier WT (1996) Fumonisins as Potential Causes of Kidney Disease. Toxin Reviews 15:273-292

Castegnaro M, Canadas D, Vrabcheva T, Petkova-Bocharova T, Chernozemsky IN, Pfohl-Leszkowicz A (2006) Balkan endemic nephropathy: Role of ochratoxins A through biomarkers. Mol Nutr Food Res 50:519 – 529

Chandrajith R, Nanayakkara S, Itai K, Aturaliya TNC, Dissanayake CB, Abeysekera T, Harada K, Watanabe T, Koizumi A (2010) Chronic kidney diseases of uncertain etiology (CKDue) in Sri Lanka: geographic distribution and

environmental implications. Environ Geochem Health. Doi:10.1007/s10653-010-9339-1
Domijan A-M, Peraica M, MArkov K, Fuchs R (2009) Urine Ochratoxin A and sphinganine/sphingosine ratio in residents of the endemic nephropathy area in Croatia. Arh Hig Rada Toksikol 60:387-393
Duarte S, Bento J, Pena A, Lino CM, Delerue-Matos C, Oliva-Teles T, Morais S, Correia M, Oliveira MB, Alves MR, Pereira JA (2010) Monitoring of ochratoxin A exposure of the Portuguese population through a nationwide urine survey - Winter 2007. Science of the Total Environment 408:1195-1198
Fazekas B, Tar A, KOVÁCS M (2005) Ochratoxin A content of urine samples of healthy humans in Hungary. Acta Veterinaria Hungarica 53: 35-44
Glahn RP, Van Campen D, Dousa TP (1994) Aflatoxin B1 reduces Na(+)-P(i) co-transport in proximal renal epithelium: studies in opossum kidney (OK) cells. Toxicology 92:91–100
Gong Y, Hounsa A, Egal S, Turner PC, Sutcliffe AE, Hall AJ, Cardwell K, Wild CP (2004) Postweaning Exposure to Aflatoxin Results in Impaired Child Growth: A Longitudinal Study in Benin, West Africa. Environ Health Perspect 112:1334-1338
Illeperuma OA, Dharmagunawardhane HA, Herarh KPRP (2009) Dissolution of aluminium from substandard utensils under high fluoride stress: A possible risk factors for chronic renal failures in the North-Central Provice. Journal of the National Science Foundation of Sri Lanka 37:219-222
Koizumi A, Harada K, Inoue K, Hitomi T, Yang H-R, Moon C-S, Wang P, Hung N, Watanabe T, Shimbo S, Ikeda M (2009) Past, present, and future of environmental specimen banks. Environ Health Prev Med 14:307-18
Malir F, Ostry V, Cernia M, Kacerovsky J, Roubal T, Skarkova J, Brndiar M, Fixa P (2004) Monitoring the important mycotoxin biomarkers (ochratoxin A, aflatoxin M1) in the Czech population. Cas Lek Cesk 143:691-6
Parsons D, Casado MR, Magan N, Dyer C, Weightman R (2007) Development of representative sampling plans for mycotoxins in foods using distribution modeling. Final report to the UK Food Standards Agency Project CO3055: Wolverhampton, ADAS UK Ltd.
Petzinger E and Weidenbach A (2002) Mycotoxins in the food chain: the role of ochratoxins. Livestock Production Science 76:245-250

211 Ringot D, Changoa A, Schneider YJ, Larondelle Y (2006) Toxicokinetics and
212 toxicodynamics of ochratoxin A, an update. Chemico-Biological Interactions
213 159:18-46

214 Sauvant C, Holzinger H, Mildenberger S, Gekle M (2005) Exposure to
215 nephrotoxic ochratoxin A enhances collagen secretion in human renal proximal
216 tubular cells. Mol Nutr Food Res 49:31-7

217 Silva LJ, Pena A, Lino CM, Fernandez MF, Manes J (2010)Fumonisin
218 determination in urine by LC-MS-MS. Anal Bioanal Chem 396:809-16
219 Wanigasuriya KP, Peiris H, Ileperuma N, Peiris-John RJ, Wickremasinghe R
220 (2008) Could ochratoxin A in food commodities be the cause of chronic kidney
221 disease in Sri Lanka? Trans R Soc Trop Med Hyg 102:726-728
222 Zepnik H, Volkel W, Dekant W (2003) Toxicokinetics of the mycotoxin
223 ochratoxin A in F 344 rats after oral administration. Toxicology and Applied
224 Pharmacology 192:36-44

225

226

227

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

Disease stages	Sex male/female (Total)	Age (yr) mean (range)
Stage 1 (slight)	3/4	24.14 (9–40)
Stage 2 (mild)	6/1	48 (39–59)
Stage 1–2 (early stage)	9/5 (14)	36.07 ± 15.19‡
Stage 3 (moderate)	3/3	41 (11–60)
Stage 4 (severe)	3/3	47.5 (35–58)
Stage 5 (end stage)	3/2	49.00 (30–65)
Stage 3–5 (late stage)	9/8 (17)	45.65 ± 14.90
Total (CKDue patients)	18/13	41.32 ± 15.55 (9–65)
Relatives of CKDue patients	2/4	20.67 (6–34)
Japanese controls	0/4	45.25 (42–53)

229
230

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)	ND–0.800(6)	ND–734.00	0.013–0.360 (7)	17.63–93.90	ND–0.042 (4)	ND–0.14
	Mean	0.359	230.21	0.044	39.67	<MDL	<MDL
	GM	0.092	87.41	0.035	33.33	<MDL	<MDL
Stage 2 (*n* = 7)	Range (n>MDL)	ND–0.037 (5)	ND–53.05	0.006–0.058 (6)	11.87–74.81	ND–0.036 (1)	ND–0.07
	Mean	0.018	19.58	0.085	65.07	-	-
	GM	0.012	10.95	0.039	35.95	-	-
Stage 1–2	GM	0.033	30.93	0.037	34.62*	-	-
Stage 3 (*n* = 6)	Range (n>MDL)	ND–0.039 (4)	ND–44.74	ND–0.028 (5)	8.57–41.25	ND–0.130 (1)	ND–0.19
	Mean	0.023	25.57	0.022	21.76	-	-
	GM	0.022	18.75	0.016	19.36	-	-
Stage 4 (*n* = 6)	Range (n>MDL)	ND–0.800 (4)	ND–991.57	ND–0.019 (4)	ND–34.27	-	-
	Mean	0.140	174.82	0.016	18.75	ND	ND
	GM	0.009	12.71	0.012	17.07	-	-
Stage 5 (*n* = 5)	Range (n>MDL)	ND	ND	0.010 (4)	ND–27.06	ND	ND
	Mean	-	-	0.044	16.56	-	-
	GM	-	-	0.080	14.72	-	-
Stage 3–5	GM	0.008	10.40	0.012	17.08*	-	-
Stage 1–5	GM	0.012	17.01	0.020	23.50	-	-
Relatives controls (*n* = 6)	Range (n>MDL)	0.020–0.800 (6)	5.9–1000.00	0.032–0.223 (6)	28.63–278.00	ND–0.093 (1)	ND–0.14
	Mean	0.298	249.09	0.104	88.95	-	-
	GM	0.112	84.12	0.085	63.52	-	-
Japanese controls (*n* = 4)	Range (n>MDL)	ND	ND	0.005–0.012 (4)	4.4–19.40	ND	ND
	Mean	-	-	0.007	9.69	-	-
	GM	-	-	0.007	8.14	-	-

ND: not detected; MDL: method detection limit; GM: geometric mean

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

239 **Table 3.** Urine mycotoxin level in other countries

Mycotoxin Type	Detection rate	Mean (range)	Study subjects	Country	Reference
AFL	61.29%	17.0 (ND–991.6) ng/gCr	CKDue patients	Sri Lanka	Present study
	58%	391.0 (19.0–19,219.0) pg/g Cr	General population	Czech Republic	(Malir et al. 2004)
OT A	100%	37.1 (12.4–360.0) pg/mL	CKDue patients (early stage)	Sri Lanka	Present study
	88.24%	12.0 (ND–58.2) pg/mL	CKDue patients (late stage)	Sri Lanka	Present study
	100%(n=6)	85.0 (32.0–223.0) pg/mL	Relatives of CKDue patients	Sri Lanka	Present study
	61%	13.0 (6.0–65.0) pg/mL	Healthy individuals	Hungary	(Fazekas et al. 2004)
	43%	7.0 (5.0–15.0) pg/mL	Endemic nephropathy	Croatia	(Domijan et al. 2009)
	92.20%	22.0 (ND–69.0) pg/mL	General population	Portugal	(Duarte et al. 2010)
	88%	50.8 (1.0–330.0) pg/mL	Endemic nephropathy	Bulgaria	(Castegnaro et al. 2005)
	97.6%	191.7 (1.0–191.0) pg/mL	Endemic nephropathy	Bulgaria	(Castegnaro et al. 2005)
FB	19.4%	(ND–130.0 pg/mL)	CKDue patients	Sri Lanka	Present study
	0% (LOD = 5 ng/mL)		General population	Portugal	(Silva et al. 2008)
	75%	70.1 (ND–9312.0) pg/mL	General population	Mexico	(Gong et al. 2008)

240

241