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Brief Report

Neonicotinoid concentrations in urine from chronic kidney disease patients in the North Central Region of Sri Lanka

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Abstract: Neonicotinoid concentrations in urine from chronic kidney disease patients in the North Central Region of Sri Lanka: Risako KABATA, *et al*. Department of Health and Environmental Sciences, Graduate School of Medicine, Kyoto University—Objectives: Neonicotinoid insecticides have been widely used around the world since the 1990s. Reports have been made since the 1990s of rice paddy farmers in the North Central Region (NCR) of Sri Lanka suffering from chronic kidney disease with unknown etiology (CKDu). A preliminary evaluation of the exposure of local farmers in the NCR of Sri Lanka to neonicotinoids was performed. **Methods:** We analyzed neonicotinoid and neonicotinoid metabolite concentrations in spot urine samples. We selected 40 samples, 10 from farmers with CKDu and 10 from controls from each of two areas, Medawachchiya and Girandurukotte. **Results:** Imidacloprid and desmethyl-acetamiprid were found at significantly higher concentrations in the control samples (with medians of 51 ng/l and 340 ng/l, respectively) than in the CKDu samples (medians of 15 ng/l and 150 ng/l, respectively) when the results were not adjusted for the creatinine contents. None of the six compounds that were measured in the urine samples were found at significantly higher concentrations in the CKDu samples than in the control samples. None of the neonicotinoid concentrations in the samples analyzed in this study exceeded the concentrations that have been found in samples from the general population of Japan. **Conclusions:** Farmers (both with and without CKDu) living in CKDu-endemic areas in the NCR of Sri Lanka are exposed to lower neonicotinoid concentrations than non-occupationally exposed residents of Japan.
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Key words: Chronic kidney disease, Farmer, Neonicotinoid, Sri Lanka, Urine

Neonicotinoid insecticides have been widely used around the world to treat vegetables, rice, fruit, and trees since the 1990s, because they are effective at controlling insects but have low toxicities to humans. However, the impacts of neonicotinoid insecticides on invertebrates and their predators have recently started to cause concern[1, 2]. The European Food Safety Authority reviewed the data available for three neonicotinoid insecticides (clothianidin, imidacloprid, and thiamethoxam) and evaluated the impacts of these insecticides on bees in January 2013[3]. The European Food Safety Authority also evaluated the potential for acetamiprid and imidacloprid to cause developmental neurotoxicity, and concluded that both compounds may affect human neuronal development and function[4]. Some case reports have shown that acute neonicotinoid poisoning causes acute kidney injury[5, 6].

Reports have been made since the mid-1990s of rice paddy farmers in the North Central Region (NCR) of Sri Lanka suffering from chronic kidney disease (CKD). Data from renal clinics at tertiary care hospitals have led to speculation that the prevalence of CKD in the NCR of Sri Lanka is relatively

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high[7]. Local medical specialists claim that diabetes mellitus, hypertension, and other identifiable causes of CKD are not responsible for CKD in this region, and because the CKD pathogenesis is unknown, it has been called CKD of unknown etiology (CKDu). The growing number of patients with CKDu is imposing a considerable economic burden on Sri Lankan health services because of the high cost of dialysis and transplantation.

It has generally been reported that CKDu is multifactorial. An epidemiological study revealed that there is a preponderance of CKDu in male paddy farmers and that CKDu is associated with various lifestyles factors[8]. In a previous study, we found that occupation, family history of CKDu, tobacco chewing, and a history of snakebites are independent risk factors for CKDu[9]. Familial clustering was found in some families, suggesting that genetics are involved in the development of CKDu[10]. We found a significant association between single nucleotide polymorphism in *SLC13A3* and CKD, with an odds ratio of 2.13 and a population attributable fraction of 50%, indicating a strong genetic component in susceptibility to CKDu[9]. We performed ecological investigations and found that none of 18 metals (Al, As, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr, Tl, V, and Zn) investigated were present at significantly higher concentrations in CKDu case urine samples than in control urine samples[9]. The major cause of CKDu therefore remains unknown.

Some health professionals in Sri Lanka have suggested that there could be a link between CKDu and exposure to agricultural chemicals because CKDu is highly prevalent among farmers. Exposure of CKDu patients in Sri Lanka to pesticides such as chlorpyrifos and glyphosate has been evaluated[11]. However, the exposure of farmers in the NCR to neonicotinoids has never been evaluated. The renal toxicity of neonicotinoids has been indicated in several case reports, so it is appropriate to evaluate the exposure of local people to neonicotinoids in areas in which CKDu is endemic.

In the study presented here, we conducted a small-scale case-control study for a preliminary estimation of the exposure levels of local people to neonicotinoids in CKDu endemic areas in Sri Lanka.

Materials and Methods

Ethics statement

This study was approved by the ethics committees of Kyoto University, Japan, and the Faculty of Medicine, University of Peradeniya, Sri Lanka. All human samples were obtained after receiving written informed consent from the donors, and the study was performed in accordance with the Declaration of Helsinki guidelines.

Study populations

The study populations were described in a previous study[9]. Briefly, the cases were case-series patients who were diagnosed as CKDu between January 2005 and December 2010. We excluded subjects in which CKD was a secondary complication of diabetes (defined as when the person had a history of diabetes mellitus and HbA1C (NGSP) >6.5% at the time CKD was diagnosed) or hypertension (defined as when the person had a history of hypertension and blood pressure >160/100 mm Hg untreated or 140/90 mm Hg with up to two antihypertensive agents at the time CKD was diagnosed) and cases in which CKD was caused by other known renal diseases, such as autoimmune diseases, glomerular nephritis, Fanconi syndrome, or IgA nephropathy (identified by the presence of histopathological and immunofluorescence evidence). Controls were selected arbitrarily in local communities in Medawachchiya and Girandurukotte[9]. Subjects were excluded from the control population if clinical examinations revealed hypertension (blood pressure>140/90 mmHg), proteinuria, glycosuria, HbA1C>6.5%, serum creatinine>1.2 mg/d*l*, or alpha-1-microglobulin>15.5 mg/*l*.

We selected randomly 10 case and control urine samples respectively from those collected previously in Medawachchiya and Girandurukotte[9]. The selection rates were 6.25% for the cases (n=160) in Medawachchiya, 6.62% for the cases (n=151) in Girandurukotte, 7.30% for the controls (n=137) in Medawachchiya, and 6.71% for the controls (n=149) in Girandurukotte.

Urine samples

Spot daytime urine samples were collected from the 40 subjects between April and August 2011. The estimated glomerular filtration rate (eGFR) was obtained using the modified diet in renal disease formula (glomerular filtration rate (m*l*/(min × 1.73 m^2)) =186 × serum $creatinine^{-1.154}$ × $age^{-0.203}$), which is based on the serum creatinine levels.

Estimation of daily excreted amounts of neonicotinoids in urine

To estimate the daily excretion in the urine, we divided the concentration of neonicotinoids in urine (ng/*l*) by the 1.5-fold creatinine concentration in urine (g/*l*) on assumption that daily excretion of creatinine in urine was 1.5 g for males[13].

Chemicals and reagents

We analyzed six compounds, five of which were mother compounds (acetamiprid, clothianidin, dinote-

furan, imidacloprid, and thiamethoxam) and the other a metabolite (desmethyl-acetamiprid). Imidacloprid currently accounts for approximately 41.5% of the global neonicotinoid market in terms of sales, and this is followed by thiamethoxam (23.8%), clothianidin (16.7%), and acetamiprid (10.5%)[12]. Dinotefuran accounts for approximately 3% of the global neonicotinoid market[12]. The major neonicotinoid compounds were therefore included in this study.

Acetamiprid, imidacloprid, and thiamethoxam were obtained from AccuStandard (New Haven, CT, USA). Clothianidin and dinotefuran were purchased from Wako Pure Chemical Industries (Osaka, Japan). Desmethyl-acetamiprid was obtained from Sigma-Aldrich (St. Louis, MO, USA). Isotope-labeled acetamiprid (acetamiprid-d6) was purchased from Hayashi Pure Chemical Ind. (Osaka, Japan). Acetamiprid-d3, clothianidin-d3, imidacloprid-d4, and thiamethoxam-d4 were obtained from Dr. Ehrenstorfer Inc. (Augsburg, Germany). Dinotefuran-d3 was purchased from @rtMolecule (Poitiers, France). All reagents were of analytical grade.

Extraction of neonicotinoids from urine samples

A 1 ml aliquot of a urine sample and a surrogate recovery standard (containing 0.2 ng each of acetamiprid-d3, clothianidin-d3, imidacloprid-d4, and thiamethoxam-d4 and 2 ng of dinotefuran-d3) were mixed together and then loaded onto a column containing diatomaceous earth (InertSep K-solutes, 2 m*l*; GL Sciences, Tokyo, Japan). The analytes were eluted 10 min after loading had been performed by adding 25 m*l* dichloromethane over a period of 2 min. The volume of the eluate was decreased to approximately 10 m*l* by rotary evaporation and then decreased to approximately 1 m*l* under a stream of nitrogen. The solution was then cleaned by passing it through a Supelclean ENVI-Carb-II/PSA SPE cartridge (A 500 mg, B 500 mg; Sigma-Aldrich). The analytes were eluted from the column with 10 m*l* of 20% (v/v) dichloromethane in acetonitrile over a period of 10 min. The solution was evaporated to dryness under a stream of nitrogen, and then the residue was dissolved in 30% ethanol in water.

Analysis

The extracts were analyzed using a Shimadzu Nexera System with a cation-mode atmospheric pressure electrospray interface (Shimadzu Corporation, Kyoto, Japan) and a Triple Quad 6500 mass spectrometer (AB SCIEX, Framingham, MA, USA). The analytes were separated using an Atlantis T3 column (100 mm long, 2.1 mm i.d., 3 μm particle diameter; Waters, Milford, MA, USA) maintained at 40°C. The injection volume was 10 μl, and the injection rate was 200 μl/min. A gradient elution program was used, with two mobile phases, 0.1% formic acid in 10 mM ammonium acetate in water and acetonitrile. Two product ions of each analyte were measured using a multiple-reaction monitoring program in which the parameters were optimized for each analyte. The instrument detection limit was defined as the mass of the analyte of interest required to give a peak with a signal-to-noise ratio of 3. The mean recoveries of the analytes (in samples fortified with 1 ng of the analytes) were 64–100%. The largest coefficient of variation was 21%. A stock solution was diluted to make calibration standards, and these were analyzed to prepare a calibration curve with at least seven points for each analyte.

Statistics

All of the statistical procedures were performed using the JMP Pro 11 software (SAS Institute, Inc., Cary, NC, USA). A p value of <0.05 was considered significant. Each concentration below the detection limit was given a value of zero in the statistical analyses. It has previously been found that humans (n=12) who had orally ingested 5 μg doses of acetamiprid-d6, clothianidin-d3, dinotefuran-d3, and imidacloprid-d4 (all labeled with deuterium) excreted 30.7 ± 15.6% of the acetamiprid as desmethyl-acetamiprid, 63.7 ± 14.6% of the clothianidin, 92.8 ± 22.1% of the dinotefuran, and 12.7 ± 7.0% of the imidacloprid in urine within 4 days (unpublished data, Harada *et al*., 2015). Acetamiprid has been found to be rapidly metabolized to desmethyl-acetamiprid by humans (unpublished data, Harada *et al*., 2015). Half-lives of 0.58. 0.23, 0.17, and 1.45 d have been found in humans for clothianidin, desmethyl-acetamiprid, dinotefuran, and imidacloprid, respectively (unpublished data, Harada *et al*., 2015). We have also previously shown that, under steady-state conditions, the amounts of acetamiprid, clothianidin, dinotefuran, and imidacloprid that a human is exposed to each day can be estimated from the amounts of desmethyl-acetamiprid, clothianidin, dinotefuran, and imidacloprid, respectively, excreted in urine over 24 hours (unpublished data, Harada *et al*., 2015).

Results and Discussion

The general characteristics of the participants are given in Table 1. Each of the 40 male participants had resided in either Medawachchiya or Girandurukotte for at least 10 years. The mean age of the participants was 45.5 years, and the mean ages of the cases and controls were not significantly different. Farmers made up 85% of the cases and 35% of the controls. The mean creatinine concentration in urine for all of the participants was 0.943 g/*l*, and

Table 1. Characteristics of participants according to the presence of CKDu

Variables	Total	CKDu cases	Controls	*p* (*t*-test)
Number	40	20	20	—
Age (yr) (mean [SD])	45.5 [8.67]	43.6 [9.37]	47.3 [7.71]	0.18
Male sex (n)	40	20	20	—
Farmers (%)	60	85	35	—
Serum creatinine (mg/d*l*) (mean [SD])	1.31 [0.550]	1.74 [0.454]	0.866 [0.0986]	<0.0001
Urinary creatinine (g/*l*) (mean [SD])	0.943 [0.618]	0.782 [0.482]	0.110 [0.705]	0.10
eGFR (m*l*/(min × 1.73 m^2)) (mean [SD])	76.2 [30.1]	50.2 [17.3]	102 [11.7]	<0.0001

SD, standard deviation; eGFR, estimated glomerular filtration rate.

the concentrations in the case and control samples were not significantly different. The mean creatinine concentrations in serum were 1.74 mg/d*l* for the cases and 0.866 mg/d*l* for the controls, and these means were significantly different ($p<0.0001$). The mean eGFRs were 50.2 m*l*/(min × 1.73 m^2) for the cases and 102 m*l*/(min × 1.73 m^2) for the controls, and these means were significantly different ($p<0.0001$).

The neonicotinoid concentrations in the urine samples from the cases and controls are shown in Table 2. Clothianidin, desmethyl-acetamiprid, imidacloprid, and thiamethoxam were detected, but none of the other analytes were detected (the detection limits were 5 ng/*l* for acetamiprid and desmethyl-acetamiprid, 20 ng/*l* for clothianidin, and 10 ng/*l* for dinotefuran, imidacloprid, and thiamethoxam). Imidacloprid and thiamethoxam are used to control invasive alien pests in Sri Lanka[14].

The imidacloprid and desmethyl-acetamiprid concentrations (unadjusted for creatinine) were significantly different in the case and control samples (determined using nonparametric analysis), but the concentrations of the other two analytes that were detected were not significantly different in the case and control samples. The median imidacloprid concentration in the control samples was 51 ng/*l*, which was significantly higher than the median concentration in the case samples (15 ng/*l*) ($p=0.02$, Wilcoxon). The median desmethyl-acetamiprid concentration in the control samples was 340 ng/*l*, which was significantly higher than the median concentration in the case samples (150 ng/*l*) ($p=0.04$, Wilcoxon). However, the concentrations in the case and control urine samples were not significantly different when the daily neonicotinoid excretions were expressed as nanograms per 1.5 grams of creatinine. The disappearance of the significant differences between the case and control samples when the daily excretions were estimated from creatinine concentrations suggests that the differences between the unadjusted concentrations were caused by the CKD patients having impaired abilities to concentrate urine.

In supplementary analyses, no significant differences were found between the neonicotinoid concentrations in the samples from non-farmers and farmers in the control group (Supplementary Table 1). We also found no significant differences between the neonicotinoid concentrations in the samples from the farmers in the control and case groups (Supplementary Table 2), indicating that there is no association between exposure to neonicotinoids and CKDu.

This is the first study in which the exposure of CKDu patients in the NCR of Sri Lanka to neonicotinoids has been evaluated. The case and control samples contained lower concentrations of neonicotinoids than have been found in samples from the general population of Japan[15].

The present study had several limitations. First, it was only a small case-control study. Second, we do not currently have enough evidence to conclude that a consistent proportion of thiamethoxam intake is excreted in urine and that the daily exposure amount of thiamethoxam can be estimated from the amount of this chemical excreted in 24-h urine under steady-state conditions. Third, acetamiprid and dinotefuran have short half-lives, so the time the urine samples are collected is important. It would be desirable to investigate information related to exposure more rigorously than we were able to achieve, and it would also be desirable to take 24-h urine samples rather than spot urine samples. However, our findings were strengthened by evidence that acetamiprid and dinotefuran are predominantly eliminated from the body by being excreted in urine, the excretion rates as percentages of the daily intakes being 30.7% for acetamiprid and 92.8% for dinotefuran. Lastly, we could not find appropriate statistical data for the use of neonicotinoids in Sri Lanka. However, this study is important because we measured neonicotinoid concentrations in samples provided by residents of areas in which CKDu is endemic.

In conclusion, farmers (both with and without

Table 2. Urinary neonicotinoid concentration in cases and controls

Component (ng/*l*) unadjusted for creatinine	Controls (n=20)			Cases (n=20)			*p* (Wilcoxon)
	n>LOD (%)	Min-max	25%/50%/75%	n>LOD (%)	Min-max	25%/50%/75%	
Acetamiprid	0 (0)	—	—	0 (0)	—	—	—
Clothianidin	4 (20)	ND-160	ND/ND/ND	3 (15)	ND-230	ND/ND/ND	0.76
Desmethyl-acetamiprid	20 (100)	86-1500	170/340/470	20 (100)	14-1300	50/150/380	0.04
Dinotefuran	0 (0)	—	—	0 (0)	—	—	—
Imidacloprid	17 (85)	ND-410	22/51/83	13 (65)	ND-460	ND/15/31	0.02
Thiamethoxam	8 (40)	ND-430	ND/ND/24	5 (25)	ND-350	ND/ND/11	0.35
Component (ng/day)[a] estimated from creatinine concentration	Controls (n=20)			Cases (n=20)			*p* (Wilcoxon)
	n>LOD (%)	Min-max	25%/50%/75%	n>LOD (%)	Min-max	25%/50%/75%	
Acetamiprid	0 (0)	—	—	0 (0)	—	—	—
Clothianidin	4 (20)	ND-100	ND/ND/ND	3 (15)	ND-320	ND/ND/ND	0.82
Desmethyl-acetamiprid	20 (100)	110-2500	310/620/1000	20 (100)	40-1900	200/290/710	0.09
Dinotefuran	0 (0)	—	—	0 (0)	—	—	—
Imidacloprid	17 (85)	ND-2600	29/51/140	13 (65)	ND-610	ND/31/110	0.25
Thiamethoxam	8 (40)	ND-270	ND/ND/41	5 (25)	ND-490	ND/ND/14	0.37

LOD, limit of detection; SD, standard deviation; ND, not detected. [a]Daily excretion in urine was calculated assuming that the amount of creatinine excreted each day was 1.5 g for males[13].

CKDu) living in areas in the NCR of Sri Lanka in which CKDu is endemic are exposed to lower neonicotinoid concentrations than non-occupationally exposed residents of Japan. Our results did not support the involvement of neonicotinoid insecticides in the pathogenesis of CKDu.

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