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Urinary melamine excretion and increased markers of renal tubular injury in patients with calcium urolithiasis: A cross-sectional study☆

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ABSTRACT

Environmental low-dose melamine exposure has been associated with urolithiasis risk in adults, but it is unclear if this exposure can cause early renal damage. This cross-sectional study investigated the association of this exposure and early renal damage in patients with calcium urolithiasis. We recruited patients diagnosed with upper urinary tract calcium urolithiasis from three hospitals in southwestern Taiwan between November 2010 and January 2015. All patients completed a structured questionnaire and provided one-spot urine samples for the measurement of melamine level and markers of early renal injury, including N-acetyl b-D-glucosaminidase (NAG), β2-microglobulin (β2-MG), and microalbumin. We used urinary melamine levels as an indicator of environmental melamine exposure. A total of 309 patients (mean age of 54.7 ± 12.8 years) were studied. Median urinary melamine level (μg/mmol Cr) was 1.26 (interquartile range 0.48–3.29). A significant and positive correlation was found between urinary melamine concentration and urinary NAG levels (Spearman correlation coefficient, r = 0.157, p = 0.006, n = 309). With urinary melamine levels categorized into quartiles, multivariate regression results showed the same relationship, particularly in those with first stone episode. In this group, patients with the highest quartile of urinary melamine concentration had a 3.95-fold risk (95% confidence interval = 1.43–10.94) of high NAG levels (dichotomized by median), compared to the lowest quartile after adjustment. No association was found between urinary melamine concentration and urinary microalbumin levels. In conclusion, urinary melamine is significantly associated with urinary marker of early renal tubular injury, NAG, in urolithiasis patients, especially ones with first stone episode.

1. Introduction

The chemical melamine is ubiquitously present in our environment, even after the 2008 toxic milk scandal in China (Ingelfinger, 2008; Bhalla et al., 2009; Gossner et al., 2009). Around the globe, studies have found melamine contamination to be more pervasive in daily-use foodstuffs than originally thought (Bhalla et al., 2009; Gossner et al., 2009). A series of our earlier studies have found environmental long-term low-dose melamine

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exposure, by using the proxy of urinary melamine levels, can increase the risk of urolithiasis formation in adults (Wu et al., 2010, 2014; Liu et al., 2011; Liu et al., 2012).

What exact mechanisms underlying the effect of chronic low-dose melamine exposure on the development of urinary tract stones and early renal damage are still elusive. Two possible mechanisms have been proposed (Wu et al., 2010; Liu et al., 2011): (1) Melamine could possibly serve as a nidus to promote stone formation in early stages and (2) chronic melamine exposure could possibly induce reactive oxidative stress in renal parenchyma and then cause renal damage, particularly renal tubular injury, promoting stone formation. The first mechanism has been indirectly confirmed by Poon and his colleagues who found that melamine significantly enhanced calcium oxalate precipitation in an *in-vitro* study (Poon et al., 2012) and by our early studies in which we detected melamine in human stone specimens (Liu et al., 2012; Wu et al., 2014). Our later *in-vitro* study found melamine induced renal tubular injury via oxidative stress and transforming growth factor-β in human renal proximal tubular HK-2 cells (Hsieh et al., 2012). Recently, we also found a significant positive correlation between urinary melamine levels and urinary biomarkers of renal tubular injury in a study of workers at a melamine tableware manufacturing factory (Wu et al., 2015). Whether this relationship also exists in other highly susceptible populations, such as patients with urolithiasis (Wu et al., 2010; Liu et al., 2011), needs further clarification. Therefore, we conducted a multihospital-based cross-sectional study in patients with upper urinary tract calcium urolithiasis, the most common type of adult urolithiasis (Chou et al., 2007), to further elucidate the association between melamine measured in urine specimens and markers of early renal injury. Whether the episode of stone formation (first *vs.* recurrent) can modify the above two was also examined.

2. Materials and methods

2.1. Subjects

This study followed the guidelines of STROBE (von Elm et al., 2007) (see Supplementary Materials Table S1). Patients diagnosed as having upper urinary tract calcium urolithiasis were recruited from Kaohsiung Medical University-affiliated hospitals: Kaohsiung Medical University Hospital (KMUH), Kaohsiung Municipal Hsiao-Kang Hospital (KMHKH) and Pingtung Hospital (PH) in southwestern Taiwan between November 2010 and January 2015. The selection criteria of eligible subjects have been described in detail previously (Liu et al., 2007, 2009, 2010, 2011). Briefly, eligible subjects were individuals aged ≥20 years who had been diagnosed with upper urinary tract urolithiasis by ultrasonography and/or radiography and who had provided stone specimens confirmed to have calcium components by infrared spectroscopy analysis (Spectrum RX I Fourier Transform-Infrared System, PerkinElmer, USA). None of the participants was found by X-ray to have radiolucent stones or by clinical evaluation to have cystine or uric acid stones. Subjects were excluded if they had a history of chronic urinary tract infection, renal failure, chronic diarrhea, gout, renal tubular acidosis, primary and secondary hyperparathyroidism, or cancer. We also excluded anyone who had regularly taken diuretics, potassium citrate, vitamin D or calcium supplements more than once per week within six months prior to the diagnosis of calcium urolithiasis or interview. All study subjects resided in southwestern Taiwan. The study protocol was approved by the Institutional Review Board of KMUH. Each participant provided signed informed written consent.

2.2. Data collection

All participants were interviewed by trained researchers using a structured questionnaire to collect detailed demographic data, medical history, and a history substance use (alcohol, cigarette, and betel quid) (Liu et al., 2007, 2009, 2010, 2011). Subjects were defined as alcohol drinkers, cigarette smokers or betel quid chewers if they had regularly consumed any alcoholic beverage ≥1 times per week, had smoked ≥10 cigarettes per week, or had chewed ≥7 betel quids per week, respectively, for at least 6 months. Current users were those who were still using any of these substances within one year before diagnosis of calcium urolithiasis or the interview (Liu et al., 2009, 2011).

Clinical information including total number of stone episodes was also collected by questionnaire and further reviewed using the patients' medical charts by one urologist (Liu CC), who was unaware the exposure of interest: urine melamine concentrations. Body weight (kg) and body height (cm) were measured with participants standing in light street clothes. Body mass index (BMI (kg/m^2)) was calculated as body weight divided by square body height.

2.3. One-spot urine samples for measurement of melamine and markers of early renal injury

After overnight fasting, all participants provided a one-spot overnight urine sample (midstream first void) for biochemical measurement of melamine and markers of early renal injury, before any intervention for urolithiasis. Melamine levels in 1-spot overnight urine sample were proved to predict the previous 24-hour total urinary melamine excretions in our earlier studies (Liu et al., 2011; Lin et al., 2013). After collection of one-spot urine, these samples were aliquoted into four samples: one for routine urinary analysis, another for melamine, another for markers of early renal injury, including microalbumin, N-acetyl b-D-glucosaminidase (NAG), and β2-microglobulin (β2-MG), and the other was stored at a −80 °C freezer for future analysis.

Urinary melamine was measured using a isotopic liquid chromatography/tandem mass spectrometry method (LC-MS/MS) (API4000Q, Applied Biosystems/MDS SCIEX, Concord, Canada) (Wu et al., 2010; Liu et al., 2011), and urinary microalbumin, NAG, and β2-MG were measured by enzyme-linked immunosorbent assay (Wu et al., 2015). The methods used to analyze melamine and markers of early renal injury are described comprehensively elsewhere (Wu et al., 2010, 2015; Liu et al., 2011). Briefly, for the melamine measurement, the elute of 1 ml urine sample collected from an Oasis MCX SPE cartridge (Waters, Malford, MA) was dried under nitrogen gas. The residues were reconstituted in 200 ml mobile phase and subjected to LC-MS/MS for analysis. The MDL in urine was 0.4 ng/ml. Thirty-one (10.0%) out of 309 measurements in 309 subjects were below MDL and treated as half, 0.2 ng/ml. Urinary creatinine was analyzed using spectrophotometry (U-2000; Hitachi, Tokyo, Japan) at a wavelength of 520 nm to measure the creatinine–picrate reaction. Urinary melamine levels were expressed as ng/ml and μg/mmol creatinine (Cr) without and with the correction of urinary creatinine, respectively.

For the measurement of markers of early renal injury, the assay kits included microalbumin kit/ALB-TIA "SEIKEN" X1 (Denka Seiken, Tokyo, Japan), NAG assay kit (Diazyme Laboratory, Poway, CA), and N Latex β2-microglobulin assay (Siemens Healthcare Diagnostics, Marburg, Germany). The MDLs were 4 mg/l for microalbumin. Eighty-eight (28.5%) out of 309 measurements were below MDL and treated as half, 2 mg/l. For urine β2-MG, only 16 (5.2%) out of 309 were detectable, whereas urinary NAG measurements were all detectable. The measurements of melamine and biomarkers of early renal injury were performed by two different

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