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Metabolomics evaluation of hydroxyproline as a potential marker of melamine and cyanuric acid nephrotoxicity in male and female Fischer F344 rats

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ABSTRACT

Following kidney failure in domesticated pets in the US and kidney issues requiring hospitalization with some deaths in children in China, investigators determined the cause was adulteration of pet foods and baby formula with melamine. It has since been noted that exposure of rats to melamine and cyanuric acid forms melamine cyanurate crystals in the kidney leading to acute nephrotoxicity. This metabolomics study aimed to identify biomarkers of melamine and cyanuric acid-induced renal injury. Male and female Fischer 344 rats were fed a diet fortified with varying doses of melamine and cyanuric acid for 28 days. Analysis of urinary amino acids showed hydroxyproline was increased in both sexes in a manner consistent with the clinical chemistry and histopathology data; most prominent when total urine output was taken into account. Furthermore, rats with the highest levels of urinary hydroxyproline were the only rats that exhibited fibrosis within the kidney. Clinical chemistry and histopathology indicated male rats were slightly more affected than female rats following dosing with the 120 and 180 ppm formulations; hydroxyproline excretion also supports this finding. Hydroxyproline may be a noninvasive urinary biomarker for detection of acute kidney injury potentially associated with kidney fibrosis.

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1. Introduction

Melamine is a compound that is commonly used in manufacturing of plastics and flame-resistant materials. Prior to 2007, melamine was considered to be nontoxic or minimally toxic. However, in 2007, domesticated cats and dogs in the United States developed kidney failure leading to a voluntary recall of more than 100 brands of cat and dog food. Research determined that the pet foods contained melamine-tainted wheat flour exported from China as wheat gluten. Additionally, cyanuric acid, a melamine analog, was also found to be present in the pet foods. Dobson et al. (2008) showed that a combination of melamine and cyanuric acid led to the formation of insoluble crystals in the kidneys. A similar event occurred in 2004, which has also been attributed to melamine adulteration due to similarities in the autopsy reports of animals that perished in 2007 (Bhalla et al., 2009). In 2008, melamine-tainted infant formula caused serious illnesses and deaths of babies in China (Hau et al., 2009; Skinner et al., 2010; Tyan et al., 2009). In 2009, the World Health Organization (WHO) reported an estimated 51,900 children in China were hospitalized with six deaths due to melamine contamination. Children were hospitalized for urinary problems and possible renal tubular blockages and kidney stones (WHO, 2009). In both events, melamine was used as a food additive to falsely increase the apparent protein content of the pet food ingredients or formula. The high nitrogen level of melamine confers the analytical characteristics of protein molecules and is not distinguishable from actual protein sources with traditional protein assays.

Following the events of 2007 and 2008, there was a new interest in understanding the toxicity of melamine, a compound that had been considered nontoxic as noted above and had a reported LD_{50} in rats of 3.2 g/kg/bw (Melnick et al., 1984). Prior to the recent outbreaks, there was little data regarding the toxicity of melamine as it was considered relatively safe. Melamine has been shown to be rapidly excreted in the urine (Jacob et al., 2012; Mast et al., 1983) but in the presence of cyanuric acid can precipitate in distal

Abbreviations: AIC, Akaike Information Criterion; AKI, acute kidney injury; BUN, blood urea nitrogen; MS, mass spectrometry; REML, restricted maximum likelihood; UPLC/QTof, ultra performance liquid chromatography/quadrupole time-of-flight.

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renal tubules as a remarkably insoluble hydrogen-bonded complex of melamine cyanurate (Brown et al., 2007; Puschner et al., 2007). Studies have shown that dosing cats, pigs, fish, or rats with melamine or cyanuric acid alone is insufficient to cause nephrolithiasis or acute kidney injury (AKI), however, simultaneous dosing with both compounds results in crystallization in the kidney in both *in vivo* and *in vitro* studies (Brown et al., 2007; Jacob et al., 2011, 2012; Reimschuessel et al., 2008). The mortality from melamine–cyanuric acid in pet food was reported to be as high as 74% in dogs and 61% in cats (Burns, 2007).

Previous studies of drug-induced renal toxicity have shown aminoaciduria that has been attributed to renal cortical toxicity (Holmes et al., 1990, 1995), renal tubular dysfunction (Gartland et al., 1989), and impaired reabsorption due to injured proximal tubules (Maddox and Gennari, 1987; Waters et al., 2005). Recent studies addressing the combined toxicity of melamine and cyanuric acid have shown that melamine cyanurate crystals can form in the renal tubules (Puschner and Reimschuessel, 2011). Xie et al. (2010) reported a metabonomics evaluation of renal toxicity induced by melamine, cyanuric acid, or a combination of the two compounds in male, Wistar rats using an ultra performance liquid chromatography/quadrupole time-of-flight (UPLC/QTof) mass spectrometry (MS) approach. Melamine alone was shown to induce renal toxicity in a dose-dependent manner while the combination of melamine and cyanuric acid resulted in the greatest toxicity and alterations in kidney morphology. Dosing with melamine or melamine and cyanuric acid produced crystal deposition in collecting ducts of renal papilla. Metabolites related to tryptophan, polyamine, and tyrosine metabolism as well as those related to gut microflora were altered after dosing with melamine or the mixture of melamine and cyanuric acid. Therefore, it may be anticipated that dosing with melamine and cyanuric acid may show a significant increase in amino acids due to renal injury following crystal formation. In the present study, a targeted analysis of amino acids was employed to investigate whether urinary aminoaciduria is a potential marker of nephrotoxicity induced by dietary co-exposure to melamine and cyanuric acid. Furthermore, this study evaluated the effects of co-exposure to melamine and cyanuric acid in both male and female rats.

2. Materials and methods

2.1. Chemicals

Optima LC/MS grade acetonitrile and water were purchased from Fisher (Pittsburgh, PA). Formic acid was obtained from Sigma Aldrich (St. Louis, MO). The AccQ•Tag™ kit and Amino Acid Standard H were purchased from Waters (Milford, MA). Hydroxy-L-proline was from Calbiochem (San Diego, CA). NMR solvents, 1,1-difluoro-1-trimethylsilanyl methyl phosphonic acid (DFTMP) was obtained from Bridge Organics, (Vicksburg, MI) and 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS-d6) in deuterium oxide (D_2O) was obtained from Chenomx (Edmonton, Alberta, Canada).

2.2. Animal care and treatment

Rat urine samples were obtained from animals evaluated in the 28-day study reported by Gamboa da Costa et al. (2012), where a detailed description of the animal treatment, histopathological, and clinical chemistry procedures is provided. Briefly, F344 rats (12 males and 12 females per dose group, 10-weeks old) were fed *ad libitum* for 28 days with NIH-41 irradiated meal (control animals), or with NIH-41 irradiated meal containing 0, 60, 120, 180, or 240 ppm each of melamine and cyanuric acid. Twenty-four hour urine samples were collected on ice in 50 mL polypropylene tubes containing 1 mL of 1% sodium azide on days −1, 0, 1–4, 14, and 28. The total urine volume was recorded and urine stored at −80 °C prior to analysis. Kidney samples were harvested and terminal blood collected following the day 28 urine collection. Histopathology was evaluated from H&E stained kidney sections (Gamboa da Costa et al., 2012). Targeted metabolomics approaches were employed for analysis of the urine samples from a subset of the total sample group; six males and six females in each dose group were evaluated for a total of 420 urine samples. Specific amino acids analyzed included hydroxy-L-proline, L-alanine, L-arginine, L-aspartic Acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine·HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine. All amino acids measured other than hydroxy-L-proline were present in Amino Acid Standard H (http://www.thermoscientific.com/ecomm/servlet/productsdetail_11152_12826742_-1).

2.3. Targeted analysis of amino acids using AccQ•Tag derivatization

Amino acids were derivatized using AccQ•Tag™ reagents according to the manufacturer's protocol. Each reaction vial contained 210–280 nmoles of derivatization reagent. Typical values of amino acids in rat urine range from 0.05 to 2.15 nmol/μL. Briefly, 10 μL of either a standard solution of amino acids (Amino Acid Standard H), hydroxyproline or urine was combined with 70 μL of AccQ•Tag™ Ultra borate buffer and 20 μL of AccQ•Tag™ reagent in a total recovery autosampler vial. Since 10 μL of rat urine was diluted to 80 μL in buffer before the derivatization reagent was added, there was sufficient excess reagent to drive the reaction to completion. The sample pH, after dilution in buffer, was approximately 9.0 (the range required for the reaction based upon the protocol is pH 8.0–10.0). The vials were placed in a heating block set to 55 °C for 10 min. All samples and a standard mixture were derivatized in batches and analyzed the same day as prepared. The autosampler was kept at 20° to minimize hydrolysis. The steps described above were employed to ensure that the derivatization was efficient and reproducible.

Chromatographic separation was performed on a Waters Acquity UPLC system (Waters Corporation, Milford, MA), equipped with a tunable UV detector, and a Waters Xevo triple-quadrupole mass spectrometer. The separation column was a Waters AccQ•Tag™ Ultra column (2.1 mm × 100 mm, 1.7 μm particle size) at 55 °C with a flow rate at 0.7 mL/min. Eluent A was 10% AccQ•Tag™ Ultra concentrate solvent A (1:10 dilution), and eluent B was 100% AccQ•Tag™ Ultra solvent B (neat). The nonlinear separation gradient was 0–0.54 min (99.9% A), 5.74 min (90.0% A), 7.74 min (78.8% A), 8.04–8.64 min (40.4% A), 8.73–10 min (99.9% A). A VanGuard Waters pre-column (2.1 mm × 5 mm, 1.7 μm particle size) was used as a guard column. One microliter of the amino acid standard mixture, hydoxyproline or urine sample was injected for analysis. The UV detector was set at 260 nm, with a sampling rate of 20 points/s.

Select ion monitoring was used with the mass spectrometer operating in positive electrospray ionization mode for quantitation of each amino acid. The capillary voltage was 3.3 kV, cone voltage was 20 V, extractor voltage was 3 V, source temperature was 150 °C, desolvation temperature was 450 °C and desolvation gas flow was 700 L/h. The ionization energies were optimized by direct infusion of standards. Concentrations (pmol/μL) were extrapolated from a standard curve prepared by serially diluting a 250 pmol/μL solution of Amino Acid Standard H. The concentrations were multiplied by the total urine volume collected in the 24 h time period to give the total excreted for each amino acid. As noted above, hydroxyproline was also derivatized according to the AccQ•Tag™ protocol. Hydroxyproline concentrations were also extrapolated from a standard curve that was prepared daily and run with each batch. The MS parameters were optimized by direct infusion of derivatized hydroxyproline. The MRM transition monitored was m/z 302.1 > 171, which is the result of fragmentation of the ureide bond in the derivatized adduct.

2.4. NMR analysis

Samples were prepared by combining 400 μL of urine, 185 μL of sodium phosphate (NaPi) buffer (1 M, pH 7.4), 60 μL DSS in D_2O and 55 mM DFTMP in NaPi buffer. Proton (^1H) NMR spectra were acquired on a Bruker Avance spectrometer operating at 600.133 MHz for proton and equipped with a triple resonance cryoprobe. Water suppression was achieved through application of the Bruker "noesypresat" pulse sequence, which irradiates the water resonance during a delay time (d1 = 2.5 s) and a mixing time (d8 = 100 ms). For each sample, 32 scans were collected into 65,536 data points. A spectral width of 9615.39 Hz was utilized with an acquisition time of 3.41 s.

Spectra were processed using ACD/NMR Workbook (Toronto, Canada) and spectra exported for quantification of select metabolites. Creatinine was quantified in order to normalize the LC–MS amino acid data using the Chenomx NMR Suite (Chenomx, Edmonton, Alberta, Canada), which has a database of >250 compounds. Failure to account for the differences in water consumption, which was especially high at the 240 ppm dose level due to the diuretic effect of melamine greatly impacted the dataset and made it appear that many metabolites were decreased following dosing due to dilution of the urine samples.

2.5. Statistical analysis

All statistical analyses were performed using the 'nlme', 'lme4' and 'lattice' packages of R statistical software (http://www.R-project.org). The experimental set-up was a repeated measures design with rats randomized into different dose groups of melamine and cyanuric acid. Urine samples were collected at multiple time points from each rat during the course of the experiment. Therefore, levels of hydroxyproline, either normalized to creatinine or to total urine volume, were analyzed for five different doses of melamine and cyanuric acid across four different time points. A linear mixed effects model was used for the analysis with the dose