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## Diagnostic tools for detection of intoxication by melamine and its analogue

**研究背景：**2008年揭露的三聚氰胺毒奶粉事件引起其對兒童健康潛在的長期影響的關注，無論是對於在子宮內就已暴露於三聚氰胺的胎兒或曾接觸三聚氰胺的兒童。相關的人類研究缺乏，限制了我們對三聚氰胺可能造成的損傷的評估。

**研究目標：**研發新的診斷工具，並提供實驗室技術支援，以協助研究人員對受影響者的不良健康影響進行研究。

**實驗設計和方法：**(1) 開發新型可靠的提取方法，從人或動物體液（血清，尿液，羊水和母乳）或組織（胎盤，結石）提取三聚氰胺和三聚氰酸，提高檢測靈敏度。(2) 引入新的生物標誌物，尿液中性粒細胞明膠酶相關脂質運載蛋白（NGAL），作為腎臟損傷早期檢測的替代標誌物。通過提高實驗室檢測能力，協助相關部門的評估，並促進對接觸三聚氰胺個體健康結果的監測計畫。

**主要觀察指標：**鑒定母體和胎兒樣本中三聚氰胺和三聚氰酸，檢測腎臟損傷早期標誌物NGAL。

**研究結果：**在接觸三聚氰胺的孕婦及其新生兒的不同生物樣本中，三聚氰胺的含量並沒有顯著增加。接觸三聚氰胺的高危兒童尿液中NGAL濃度沒有升高，表明這些兒童沒有明顯的腎臟損傷。

**結論和啟示：**運用最先進的液相色譜串聯質譜方法，本實驗室成功研發出可從各種生物樣本定量微量三聚氰胺和三聚氰酸的可靠實驗方法，為接觸三聚氰胺相關的科學及臨床研究提供堅實的基礎。

### Introduction

In September 2008, melamine contamination in milk and infant formulae raised global concerns about food safety in mainland China. The contamination resulted in an epidemic of renal calculi, especially among infants and children. According to a report from the Chinese Ministry of Health, 294 000 infants had been affected by the end of November 2008. More than 50 000 infants had been hospitalised, and six deaths were confirmed.<sup>1</sup>

In Hong Kong, melamine was also detected in other dairy and food products, and the government immediately amended the Harmful Substances in Food Regulations to establish limits for melamine in food at 1 mg/kg for milk and food for children under the age of 36 months, pregnant and lactating women. Despite the swift action on testing and recalling of the affected products, the health damage inflicted on those who frequently consumed these products is largely unknown. In addition to intake of contaminated food or infant formula, newborns and infants may be exposed to melamine antenatally via maternal-foetal transfer through placental barrier or postnatally through breast-feeding. These people may be at risk of undiagnosed renal injury. Because of the lack of human data, the possible toxic effects are uncertain.

Melamine and its by-products are ubiquitous in the living environment, but are usually present in only minute amounts. This makes detection of these compounds in specimens a challenge to laboratories. Nonetheless, accurate quantification is of utmost importance to better understanding of their toxicokinetics and health effects in human.

Our study aims to develop new diagnostic tools and provide laboratory support to investigate the potential adverse health effects on babies at risk of in-utero melamine exposure and on children with a history of melamine exposure. Specifically, we aimed to: (1) develop new reliable extraction methods/protocols

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for melamine and cyanuric acid content in human/animal body fluids (serum, urine, amniotic fluid, and breast milk) and tissue extracts (placenta +/- stones) to improve detection sensitivity; and (2) introduce a new biomarker, urine neutrophil gelatinase-associated lipocalin (NGAL), as a surrogate marker for early detection for kidney injury. This facilitates the planning of monitoring of medium- to long-term health outcomes in melamine-exposed individuals.

## Methods

This study was conducted from April 2009 to March 2011. New extraction methods for melamine and cyanuric acid content in human/rat body fluids and foetal tissue extracts were established. They entailed isotope dilution electrospray ionisation liquid chromatography-tandem mass spectrometry (LC-MS/MS). Numerous LC-MS/MS methods have been reported. Most require tedious solid phase extraction procedures to remove the sample matrix interferences in the biological samples prior to the LC-MS/MS procedure. We simplified the sample preparation procedure to obviate solid phase extraction by diluting out the biological samples with appropriate solutions and further separate the reduced sample matrix interferences by the liquid chromatography. The methods were performed on a Waters UPLC ACQUITY UPLC Xevo TQ MS/MS system (Milford, MA, USA). The limits of quantitation for melamine and cyanuric acid were markedly improved to 3 to 10 parts per billion (ppb). This provides grounds for future studies on their effects on humans.

We participated in a prospective follow-up study on Hong Kong Chinese school children with elevated urine melamine levels.<sup>2</sup> Stored urine aliquots were collected from a territory-wide cohort surveyed in 2007-08. Urine melamine was measured by the LC-MS/MS method using our novel protocol. Electrospray positive ionisation tandem MS analyses were performed using a mass-to-charge ratio of 127-85 and 127-68 as quantitative and qualitative multiple reaction monitoring for melamine, respectively. The limit of quantitation was 5 ppb and the linearity was up to 10 000 ppb. The protocol for melamine detection can be adopted for other biological samples apart from urine. No major adverse renal outcome was detected in this cohort with elevated urine melamine level.

Our methods for cyanuric acid extraction and quantitation are applicable for tissue culture and body fluids.<sup>3</sup> Cyanuric acid is a compound that may interact with melamine to produce increased renal toxicity. Electrospray negative ionisation tandem MS analyses for cyanuric acid were performed using a mass-to-charge ratio of 128-42 and 128-85 as quantitative and qualitative multiple reaction monitoring, respectively. The limit of quantitation for cyanuric acid was 10 ppb with linearity up to 10 000 ppb. Melamine cannot be converted to cyanuric acid by mammalian cells. Therefore, renal pathology involving

deposition of melamine-cyanurate crystals must have resulted from the two reactants having been consumed simultaneously. Our findings shed insights into the stone-formation mechanism and related pathophysiological processes.

Our novel methods were further adopted for other biological fluids and tissues. Rats were used to investigate gestational and lactational transfer of melamine in animal models.<sup>4,5</sup> Rat serum, amniotic fluid, breast milk, and foetal homogenate (foetus and neonatal kidney samples) were subjected to sample extraction processes and quantitation of melamine by LC-MS/MS. The processes and findings have been described in previous reports.<sup>4,5</sup> Sample recovery of the assay approached 100%. The detection limits of melamine were at 20 ppb in amniotic fluid, 50 ppb in breast milk, 5 ppb in serum and tissue homogenate.

### *Project 1: study of materno-foetal transfer of melamine*

During September to November 2008, pregnant women with a dietary history of exposure to melamine contaminated food products were recruited during their prenatal visits. Their exposure to melamine over the past few months was assessed. The extent of melamine intake per kg of body weight per day of exposure was derived ( $\mu\text{g}/\text{kg}/\text{day}$ ). Biological samples were collected from the mothers, namely 5 mL whole blood, 30 mL urine, 30 mL amniotic fluid (if available) and placental tissue (if available). After delivery, biological samples were collected from the neonates, namely 5 mL cord blood and 10 mL urine within 1 day of birth. Age-matched controls (pregnant women and their neonates) who did not have a history of consuming melamine contaminated food products were also used for comparison.

### *Project 2: basic laboratory development work for markers of kidney injury*

Children under 12 years of age with prolonged melamine-tainted milk product exposure ( $>200 \mu\text{g}/\text{kg}/\text{day}$ ) who had persistent urinary abnormalities and/or clinical features suggestive of renal diseases (eg frequency, dysuria, gross haematuria, frothy urine) were recruited from the Special Assessment Centres. Blood specimens were collected for general biochemistry and urine for urinalysis and NGAL determination. The children were followed up at regular intervals according to the standard protocol of the centres. Controls with no history of melamine-tainted milk product consumption were also recruited from local schools and special outpatient clinics. NGAL is a small protein produced in the distal nephron in kidney and its synthesis is upregulated in active renal tubular injury. In chronic kidney diseases, NGAL is a marker of disease severity as chronically damaged tubular cells produce it in great quantities. NGAL represents a real-time indicator of active kidney damage occurring in the course of renal impairment and is an independent predictor of chronic kidney disease progression.

## Results

In project 1, 152 pregnant women were recruited, 74 in the melamine-exposed group and 78 in the control group. Median daily melamine exposure for the former group was 1.5 (range, 0.1-80.3)  $\mu\text{g}/\text{kg}/\text{day}$ . All cases had a daily exposure lower than the World Health Organization tolerable daily intake of 200  $\mu\text{g}/\text{kg}/\text{day}$ . Only one subject had a daily exposure higher than the US Food and Drug Administration tolerable daily intake of 63  $\mu\text{g}/\text{kg}/\text{day}$ . Samples from 20 cases with the highest melamine exposure were further evaluated (Table). There was no significant difference in the melamine contents in all the biological samples measured. This could be due to the low level of melamine exposure in the cases, as they were recruited after recall of melamine contaminated food, and melamine is rapidly metabolised and excreted.

In project 2, an age-matched reference interval from local healthy children was used for comparison. Of 203 urine samples collected from normal controls, 101 (49.8%)

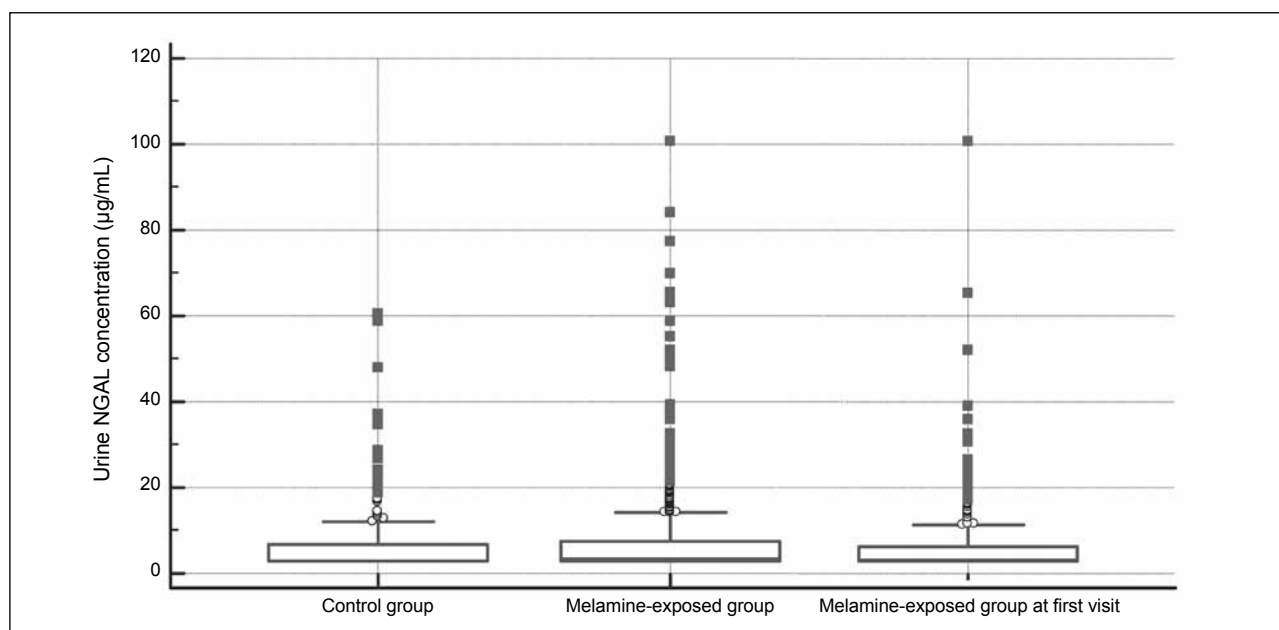
showed an undetectable urine NGAL concentration. The median level was 3.0 (interquartile range, 2.9-6.7)  $\mu\text{g}/\text{mL}$ . The reference interval established by the non-parametric percentile method was  $\leq 35.8$   $\mu\text{g}/\text{mL}$  (95th percentile) [90% confidence interval, 23.6-58.7  $\mu\text{g}/\text{mL}$ ]. Of 739 urine samples from 302 children with prolonged melamine-tainted milk product exposure, 347 (47.0%) were undetectable of NGAL. Urine NGAL concentrations were not significantly different in the melamine-exposed and control groups ( $P=0.41$ , Mann-Whitney  $U$  test, Fig). Had there been melamine-related renal adverse effects leading to increased urine NGAL, the most pronounced effect would have been detected in urine samples collected from the first visit. Urine NGAL concentrations were not significantly different in the first-visit urine samples and controls ( $P=0.98$ , Mann-Whitney  $U$  test, Fig).

## Discussion

Melamine and cyanuric acid concentrations in different biological samples are low, and their extraction for

**Table. Melamine concentration in different biological samples in the melamine-exposed and control groups**

Sample	Melamine-exposed group		Control group		P value (Mann-Whitney $U$ test)
	No. of samples	Median (range) melamine concentration	No. of samples	Median (range) melamine concentration	
Mother urine ( $\mu\text{g}/\text{mmol Cr}$ )	19	1.3 (<5-36.6)	20	1.2 (<5-74.5)	0.8259
Mother blood (ppb)	15	<5 (<5-11)	15	<5 (<5-15)	0.9682
Placenta (ppb)	20	<5 (<5-1.2)	20	<5 (<5-0.4)	0.7452
Breast milk (ppb)	5	<50	5	<50	-
Amniotic fluid (ppb)	5	<20	8	<20 (<20-306)	0.4040
Cord blood (ppb)	20	<5	20	<5	-
Neonate urine (ppb)	20	<5 (<5-18.7)	20	<5 (<5-9.7)	0.1598



**Fig. Box-and-Whisker plot of urine neutrophil gelatinase-associated lipocalin (NGAL) concentrations in the melamine-exposed (including first-visit sample) and control groups**

subsequent analysis is a challenge for laboratories. Sensitive detection of melamine and cyanuric acid is essential for studies on their effects on humans. We successfully developed protocols to quantify trace amounts of melamine and cyanuric acid in various biological samples by the LC-MS/MS with isotope-labelled internal standards.

Despite elevated urine melamine concentrations, young school children appeared to run a benign clinical course. Although melamine could reach the foetus/infant by placental/lactational transfer in rats, melamine concentrations in different biological samples collected from melamine-exposed pregnant women and their neonates were not significantly increased. Low-dose melamine intake in pregnant women did not result in significant deposition of melamine in their foetuses/infants.

There was no significant difference in urine NGAL concentration in the melamine-exposed and control groups. Urine NGAL is considered one of the most sensitive markers for renal tubular injury. The normal NGAL levels suggested absence of significant renal injury in the melamine-exposed group of infants and children.

Our laboratory development sets a foundation for studies on melamine exposure in humans. Our research findings and new diagnostic tools enable improved diagnosis, management and health care planning for

affected individuals.

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