19(S 8)

HONG KONG MEDICAL JOURNAL

香港醫學雜誌

Volume 19 ▪ Number 6 ▪ December 2013

Supplement 8

HONG KONG SUPPLEMENT 8
VOLUME 19 ▪ NUMBER 6 ▪ DECEMBER 2013

HONG KONG MEDICAL JOURNAL
香港醫學雜誌

The official publication of the
Hong Kong Academy of Medicine
and the Hong Kong Medical Association

Studies Related to Melamine Incident
Research Dissemination Reports

有關三聚氰胺事件的研究
研究成果報告

香港醫學專科學院出版社
HONG KONG ACADEMY OF MEDICINE PRESS

HONG KONG MEDICAL JOURNAL
香港醫學雜誌

Vol 19 No 6 December 2013
Supplement 8

Studies Related to Melamine Incident

Research Dissemination Reports

Research Fund Secretariat, Food and Health Bureau

Summary report on commissioned research studies related to the melamine incident

Key Messages

1. The Hong Kong SAR Government acted promptly to investigate the potential risks to the Hong Kong population resulting from melamine-tainted milk products (MTMP) originating in Mainland China.
2. The Food and Health Bureau commissioned a series of basic and applied research studies following stringent peer review.
3. Ten studies valued at over HK$6.5 million were supported.
4. Taken together, the results of this commissioned research suggest that there were no long-term adverse health consequences for Hong Kong inhabitants who had consumed MTMP.
5. The Hong Kong SAR Government has enhanced measures to ensure the continued safety of imported foodstuffs.

Background

In September 2008, melamine was found in infant formula, and infants in Mainland China were reported to have suffered from kidney stones and kidney failure after consuming such infant formula. By the end of November 2008, the Ministry of Health of the People's Republic of China, reported that 294 000 infants had been affected by melamine-contaminated infant formula, of whom more than 50 000 were hospitalised and six had died.[1]

There was public concern in Hong Kong that melamine-contaminated milk products (MTMP) might also be available locally, posing a potential risk to health, particularly in children. Responding swiftly, the Food and Health Bureau and the Hospital Authority established free screening services at 18 Designated Clinics and 8 Special Assessment Centres on 23 September 2008. The screening services were operated effectively and were able to meet the public's demands. The Designated Clinics had provided screening for 56 847 children before closing on 1 April 2009, due to declining demand, whereas the Special Assessment Centres handled 27 616 cases before closing on 9 April 2009.

A total of 15 children with renal stones suspected to be related to MTMP were reported, two by private doctors and the rest by the Hospital Authority. The notification of the last case was on 13 March 2009; no report was received thereafter.

On 26 September 2008, the Food and Health Bureau established the Expert Group on Melamine Incident, chaired by the Secretary for Food and Health, to address public health and food safety issues arising from the incident.

To address the public's concerns over the incident, the Centre for Health Protection operated a dedicated telephone enquiry hotline from 21 September 2008 to 1 April 2009 to provide an enquiry service for those who suspected themselves or their children of having consumed MTMP. Apart from answering public enquiries on the melamine incident, health advice was also disseminated to the callers when responding to their enquiries. Between 23 and 27 September 2008, the number of enquiries handled by the hotline peaked at over 1000 per day. Thereafter, the number of calls decreased continuously until termination of the dedicated hotline on 1 April 2009.

At the early stage of the incident, very little was known about the health effects of MTMP on humans; most information was based on animal studies and well-publicised overseas cases of melamine-contaminated pet food in 2007. With the development of the incident and accumulation of experience and scientific understanding, a number of studies on the health effects of melamine were published, some from the Mainland and others from local institutions. Despite limitations in methodology and data collection, they provided substantial information about the relationship between exposure to melamine and nephrolithiasis in infants and children.

Hong Kong Med J 2013;19(Suppl 8):S3-9

Research Fund Secretariat, Food and Health Bureau

Contact details:
Research Fund Secretariat, Food and Health Bureau, 9/F, Rumsey Street Multi-storey Carpark Building, 2 Rumsey Street, Sheung Wan, Hong Kong SAR, China
Tel: (852) 3150 8983
Fax: (852) 3150 8993
Email: rfs@fhb.gov.hk

Commissioned Research

Given the novel nature of melamine-related disorders, the Group recommended commissioning research studies to assess the potential medium- and long-term health effects associated with melamine exposure, including laboratory studies and basic science research. Invitations to submit proposals for projects were sent to The Chinese University of Hong Kong, Hospital Authority, and The University of Hong Kong on 18 November 2008. Priority areas for research included (1) follow-up studies of affected persons, particularly those at higher risk of adverse health outcomes, (2) laboratory testing of melamine and analogues, and (3) research involving animal models or other basic science.

All eligible applications were evaluated under a two-tiered peer review system comprising expert reviewers and an Assessment Panel. At the Assessment Panel meeting held on 20 January 2009, ten projects were recommended for funding, which addressed each of the three priority areas identified by the Group. The funds granted for the ten projects amounted to over $6.5 million. A summary of the projects is shown at the Table.

Contracts were issued in early March 2009. All projects commenced in March or early April 2009, and interim reports on the progress of each project helped to monitor and ensure the quality of each project.

Follow-up studies of affected persons

In the project MI-FU-08, Lau and Tu conducted a case-control study of Sichuan children (n=44; mean age, 25.7

Table. Summary of commissioned research studies related to the melamine incident

Project no.	Title	Funds expended (total=6 552 984)	Subjects
MI-BS-06	Melamine toxicity in rat foetuses and infants	982 760	Rats (n=10 per group) were treated with a single dose of melamine (21.4 mg/kg body weight)
MI-BS-16	Impact of melamine-tainted milk on foetal kidneys and disease development later in life	1 154 090	Mice (n=6-10 per group) were treated with both melamine and cyanuric acid (40 mg/kg/day) for 3 days
MI-BS-12	Renal and vascular function in pregnant and neonatal rats exposed to melamine and related compounds	808 417	Rats (n=6 per group) were treated with melamine (60, 300, and 600 mg/kg/day) for 3 months
MI-BS-07	Effects of melamine on urine crystallisation kinetics and cell responses	737 578	Human (tubular, gastric) and canine (tubular) cell lines
MI-BS-18	Mechanism of melamine-induced human urinary bladder carcinoma	278 760	Human bladder epithelial cells
MI-LAB-01	Urinary free to complex melamine ratio: a confirmatory test of melamine-induced urinary calculus	0	Urine samples from affected children in Mainland China with ultrasonographic evidence of urinary stones (n=30)
MI-LAB-02	Diagnostic tools for detection of intoxication by melamine and its analogue	846 059	Biological samples from melamine-exposed pregnant women (n=74) and controls (n=78). Urine samples from melamine-exposed, high-risk children (n=302)
MI-FU-08	Case-control study of Sichuan and Hong Kong children with melamine-associated renal stones: renal ultrasonography and urinary IL-8 and MCP-1 levels	462 100	Sichuan (n=44) and Hong Kong (n=22) children with melamine-associated renal stones
MI-FU-04	Prevalence of melamine exposure in Hong Kong children	300 373	Hong Kong school children with elevated urine melamine levels (n=46)
MI-FU-01	Two-year follow-up for children with melamine exposure in Hong Kong: a multicentre study	982 847	Hong Kong children with ultrasound (n=62) or urine (n=321) abnormalities

months) and Hong Kong children (n=22; mean age, 75 months) with melamine-associated renal stones. The number of renal stones in Sichuan children[2] was significantly higher than that in Hong Kong children. Urinary interleukin (IL)-8 (a marker for localised inflammation) concentration was higher in children with renal stones in Sichuan but similar in Hong Kong children with or without renal stones. About 28% of Sichuan and 48% of Hong Kong children still had renal stones at the 1-year follow-up. The authors recommended that Mainland Chinese children with persistent melamine-associated renal stones should have long-term follow-up to monitor possible renal interstitial inflammation and fibrosis. For Hong Kong children, it was unlikely that their renal stones were related to melamine and no significant clinical harm would likely to result from these stones.

In the project MI-FU-04, Kong et al conducted a prospective follow-up study (median follow-up duration, 23.5 months) in Hong Kong school children aged 6 to 20 years (n=46) with elevated urine melamine levels (urine melamine/creatinine ratio, >7.1 μg/mmol). About 9% of Hong Kong school children (n=502) had elevated urinary melamine levels. There was no significant association between milk consumption and urinary melamine level. The Hong Kong school children with high urine melamine levels appeared to have a benign clinical course in the short-term. The investigators suggest that long-term follow-up be conducted on those with persistently high urine melamine levels to identify any significant adverse clinical outcomes.[3]

In the project MI-FU-01, Lam et al conducted a multicentre 2-year follow-up study to investigate the

Summary of results	Publications (as at 30 Nov 2013)
• About 80% of melamine was found in mother's serum after administration of a single dose of melamine to pregnant rats by gavage • Melamine reached the foetuses and the kidneys of neonates through placental transfer, and was later excreted into the amniotic fluid in utero • In lactating rats, about 40% of maternal intake of melamine was transferred to breast milk • Distribution of melamine in all postnatal organs was higher than that in prenatal organs • Postnatal kidneys in early infants had the highest maximum concentration and the lowest clearance of melamine than the other postnatal organs	Chu et al.[5] 2010 Chan et al.[6] 2011 Chu et al.[7] 2013
• 34-45% of the added melamine was transferred to foetal circulation in the ex vivo human placenta perfusion model, but cyanuric acid did not influence such transfer • When the supply of drinking water was normal, no renal stones were formed in the treated mice • When drinking water was restricted, stone formation was observed	Partanen et al.[8] 2012 Peng et al.[9] 2012
• Melamine significantly reduced renal blood flow and impaired renovascular function in renal vasculature and kidney • Melamine-induced renal fibrosis and inflammatory changes • Melamine and cyanuric acid caused nephropathies in neonatal rats and impacted on postnatal rats resulting from exposure during pregnancy	Nil
• Melamine crystallised out from human urine under acidic conditions • Melamine caused the precipitation of other lithogenic salts • Citrate and bicarbonate therapy could reduce melamine crystallisation • Melamine caused physical damage to renal cells and humoral type of immune response	Poon et al.[10] 2012
• Melamine-associated crystals induced apoptosis in bladder epithelial cells, which might contribute to renal failure • Melamine stimulated the growth of bladder epithelial cells by activating mitogen-activated protein kinases • In combination with other carcinogens, melamine could facilitate the cellular transformation of bladder epithelial cells	Nil
• A pilot study showed that the liquid chromatography-tandem mass spectrometry analytical method led to negative values for complex melamine in 30-40% of cases • Pilot data showed that the hypothesised relationship was not useful	Nil
• There was no significant increase of melamine content in different biological samples collected from pregnant women and their neonates with history of low melamine exposure. • Developed protocols and quantified melamine and cyanuric acid in various biological samples from melamine-exposed pregnant women • Introduced neutrophil gelatinase-associated lipocalin as a surrogate marker for the detection of kidney injury in the urine samples from melamine-exposed, high-risk children • Demonstrated co-contamination to be the likely cause of melamine-cyanurate nephrolithiasis	Panesar et al.[4] 2010
• The number of renal stones in Sichuan children was significantly higher than that in Hong Kong children • Urinary IL-8 was higher in children with renal stones in Sichuan but similar in Hong Kong children with or without renal stones • About 28% and 48% of Sichuan and Hong Kong children respectively still had renal stones at the 1-year follow-up	Wang et al.[2] 2011
• About 9% of Hong Kong school children (n=502) had elevated urinary melamine levels • There was no significant association between milk consumption and urinary melamine level • Hong Kong school children with high urine melamine levels appeared to have benign clinical course in the short term	Kong et al.[3] 2011
• No clinically significant differences were noted between children exceeding the World Health Organization melamine tolerable daily intake and those who did not • Renal function parameters were normal in all subjects • No associations were noted between the estimated melamine exposure and the medium- to long-term adverse renal outcomes	Nil

medium- and long-term renal outcomes in children (≤12 years old) with a history of melamine exposure in Hong Kong. In those with ultrasound (n=62) or urine (n=321) abnormalities, no clinically significant differences were noted between children exceeding the World Health Organization melamine tolerable daily intake (TDI) of 0.2 mg/kg body weight and those who did not. Renal function parameters were normal in all subjects. No associations were found between estimated melamine exposure and the medium- to long-term adverse renal outcomes.

Laboratory testing of melamine and analogues

In the project MI-LAB-01, Mak et al conducted a pilot study to determine if melamine complex could be detected in urine samples from affected children in Mainland China with ultrasonographic evidence of urinary stones. Preliminary results showed that the proposed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method was unsatisfactory and no further evaluation was conducted.

In the project MI-LAB-02, Wong et al developed protocols and quantified the trace amounts of melamine and cyanuric acid present in various biological samples (serum, urine, amniotic fluid, breast milk, and placenta) collected from melamine-exposed pregnant women using a LC-MS/MS method. They also introduced neutrophil gelatinase-associated lipocalin (NGAL, lipocalin-2), a biomarker of kidney function, as a surrogate for kidney injury in urine samples from melamine-exposed high-risk children. They also demonstrated that melamine could not be converted to cyanuric acid by mammalian cells, suggesting that renal pathology involving deposition of melamine-cyanurate crystals resulted from the two compounds having been consumed simultaneously.[4]

Animal models and basic science research

In the project MI-BS-06, Wang et al investigated melamine toxicity in foetal and infant rats during and after pregnancy. Their results showed that about 80% of melamine was found in the mother's serum after administration of a single dose of melamine (21.4 mg/kg body weight) by gavage to pregnant rats. Melamine reached the foetuses and the kidneys of the neonates through placental transfer, which was later excreted into the amniotic fluid in utero. In lactating rats, melamine accumulated in mammary glands and about 40% of the maternal intake was transferred to breast milk.[5,6] Distribution of melamine in postnatal organs was higher than that in prenatal organs. Postnatal kidneys in early infants had the highest maximum concentration and the lowest clearance of melamine, compared to other postnatal organs.[7]

In the project MI-BS-16, El-Nezami et al determined the consequences of exposure of mouse foetuses to melamine and cyanuric acid. Their results showed that 34 to 45% of the added melamine was transferred to the foetal circulation in the ex vivo human placenta perfusion model, but cyanuric acid did not influence the transfer.[8] In mice treated with both melamine and cyanuric acid (40 mg/kg/day) for 3 days, no renal stones were formed when the supply of drinking water was normal. However, when drinking water was restricted, stone formation ensued, accompanied by high levels of serum urea, creatinine, urine haemoglobin, and glucose.[9]

In the project MI-BS-12, Wong et al investigated the impact of 3-month oral ingestion of melamine (60, 300, and 600 mg/kg/day) on renal and vascular function in pregnant and neonatal rats. Preliminary data demonstrated that melamine significantly reduced renal blood flow and impaired renovascular function associated with overexpression of three pro-inflammatory markers (TGF-β1, BMP1, and COX-2) within the renal vasculature. Melamine also induced renal fibrosis and inflammatory changes. Their study revealed that melamine and cyanuric acid caused nephropathies in neonatal rats and affected the health of postnatal rats exposed to melamine and cyanuric acid during pregnancy.

In the project MI-BS-07, Ng et al measured melamine and its effects on urine crystallisation kinetics and cell responses. In vitro studies revealed that melamine crystallised out from human urine under acidic conditions (pH 5.0). The presence of melamine caused the precipitation of other lithogenic salts (uric acid, calcium, oxalate, and phosphate).[10] Citrate and bicarbonate therapy significantly reduced melamine crystallisation. Such crystals caused physical damage to renal cells.

In the project MI-BS-18, Yue et al investigated the cellular effects and mechanisms of melamine and its crystals on cultured human bladder epithelial cells. They found that melamine-associated crystals induced apoptosis in bladder epithelial cells, which might contribute to the renal failure in affected subjects. They also found that melamine stimulated the growth of bladder epithelial cells by activating mitogen-activated protein kinases. In combination with other carcinogens, melamine facilitated the cellular transformation of bladder epithelial cells.

Conclusions

The commissioned projects provide a range of findings to better understand the risk to human health posed by melamine and its analogues. The effects of melamine and its analogues are wide-ranging and complex. A variety of experimental methods is needed to determine the potential adverse health effects of melamine and its analogues. Overall, the medium- and long-term health effects of MTMP in the Hong Kong population are considered minor. At least nine high-impact peer-reviewed journal publications have been produced from the research supported by the commissioned programme.

Lessons learned and perspectives

Scientific limitation of animal models

Because of insufficient human data, it is necessary to rely on toxicological studies on laboratory animals to characterise the human health risks related to melamine in food. Humans and most primates do not possess the enzyme urate oxidase, which is responsible for the conversion of uric acid to allantoin in most other mammals.[11] As a result, the melamine dosage needed to produce melamine-uric acid stones in normal rats with normal urate oxidase function may be higher than that in primates. It is therefore important to consider the normal endogenous uric acid concentration when using animal models for risk assessment. It may be informative to conduct studies to better understand melamine toxicokinetics in animal models that reflect uric acid levels in humans, especially neonates. Comparative toxicokinetics in humans and other species would also be valuable.

Need for improved analytical methods

In order to avoid adulteration with fraudulent non-protein nitrogen sources, such as melamine, development of effective and rapid methods for protein analysis that do not include non-protein nitrogen is important. Sensitive detection of real-world food and water contaminants using a portable and automated optofluidic surface enhanced Raman spectroscopy (SERS) microsystem may be useful. It exhibits up to two orders of magnitude improvement compared with conventional microfluidic SERS.[12] Using the optofluidic SERS device, melamine was detected at low concentrations, with an estimated limit of detection of 63 parts per billion. This could lead to highly sensitive and automated sensing systems for on-site detection of food and water contaminants. In addition, a simple and rapid surface-assisted laser desorption/ionisation MS approach also holds great potential for screening melamine in food (with a 5 nM detection limit).[13] Alternatively, a simple and efficient ambient ionisation method based on paper spray combined with tandem MS allows rapid detection and quantitation of various contaminants (eg clenbuterol, melamine, plasticiser, and Sudan red) in various foodstuffs (eg meat, milk, sports drinks, and chili powder).[14]

Choice of exposure measure

Several different biomarkers can be used to predict renal damage following exposure to melamine. It has been reported that renal papillary antigen-1 (RPA-1) may serve as a non-invasive urinary biomarker for the detection and monitoring of obstructive nephropathy associated with melamine and cyanuric acid exposure.[15] Alternatively, a mixture of melamine- and cyanuric acid–induced metabolites (including hydroxyproline) may be useful non-invasive urinary biomarkers for the detection of acute kidney injury, which is potentially associated with kidney fibrosis.[16,17] Further studies are required to understand the mechanism of toxicity and subtle renal alterations induced by subchronic exposure to low-dose melamine or short-term exposure to intermittent high doses. Biomarkers should be sought to evaluate the long-term effects of early-life melamine exposure in humans. Population-level urine tests may be valuable as biomarkers to indicate potential problems prior to stone formation (eg the detection of crystals of melamine-cyanuric acid or melamine-uric acid in urine).

Due to the short half-life of melamine (approximately 3 to 4 hours in mammals), very sensitive methods are required to measure the low level of melamine crystals present in urine. LC-MS/MS and gas chromatography-MS/MS can be used as confirmatory and/or screening methods due to their high selectivity and high sensitivity. However, these high-technology, high-cost methods are not cost-effective screening approaches. Development of more specific low-cost biomarkers and diagnostic techniques is needed.

Apart from nephrotoxicity, neurotoxicity may be induced by acute low-dose melamine, which affects hippocampal synaptic plasticity and behaviour in rats.[18] This selective neurotoxicity of melamine in the hippocampus may be associated with oxidative damage.[19] Further studies are warranted to assess the neurotoxicity of melamine.

Exposure to low-dose melamine in Hong Kong

The World Health Organization has established a TDI of 0.2 mg/kg body weight for melamine. This TDI is applicable to infants. The dietary exposure based on the consumption of MTMP in China was estimated to range from 8.6 to 23.4 mg/kg body weight per day, based on data provided by the Chinese Center for Disease Control and Prevention.[20] This is about 40 to 120 times the TDI, which may explain the dramatic health outcomes in Chinese infants. In contrast, all infant formulas in the Hong Kong market (84 samples) were tested by the Hong Kong Centre for Food Safety and found to comply with the legal standards. Thus, estimated potential exposure of Hong Kong infants to melamine from powdered infant formula containing adulterated milk was well below the TDI.[21]

The estimated melamine intake of the 15 Hong Kong children with renal stones was between 0.09 mg/kg/day and 0.91 mg/kg/day. The widespread and severe outbreak of melamine-related kidney stones observed in Mainland China did not occur in Hong Kong. Results from the Hong Kong screening programme suggest that large-scale and urgent screening programmes may not be informative or cost-effective for other populations who have been exposed to low-dose melamine.[22,23]

Need for continued assessment

In view of the lack of evidence to guide the government initially and the large number of severely affected children in Mainland China, a large-scale territory-wide urgent screening programme was justified at the time. As these

screening programmes showed no obvious medium- or long-term adverse effects from exposure of the Hong Kong population to MTMP, there is no compelling reason for continued follow-up of affected cases.

Research output

Apart from academic outputs, such as journal publications, the impact of the commissioned studies was further evaluated via completion of research payback questionnaires conducted 2 years after the completion of each research project. These allowed better understanding of the translation of research findings into health care service delivery and practice and contribution to health policy formulation in Hong Kong. The principal applicants of all eight projects that were completed as approved submitted a research evaluation questionnaire 2 years after project completion. Six projects (75%) resulted in publication of at least 10 peer-reviewed papers (Table). Five projects (62.5%) resulted in either gain of additional qualifications or career advancement for project team members that could be attributed in part to participation in the research. One project led to an award of additional research funding, and the results of another project led to further research by other groups. Engagement with fellow researchers and health professionals through conferences, seminars, workshops, and the general public through other media were also widely reported.

International collaboration and interdisciplinary approaches

The deliberate adulteration of food products is probably as old as the food processing and production systems themselves. With globalisation and rapid distribution systems, these incidents can have an international impact with far-reaching and sometimes fatal consequences. A well-coordinated and harmonised system to establish standards and regulations is important. The International Food Safety Authorities Network (INFOSAN) is a joint initiative between the World Health Organisation and the Food and Agriculture Organization of the United Nations. This global network includes 177 member states (including Mainland China). Each has a designated INFOSAN emergency contact point for communication between national food safety authorities and the INFOSAN secretariat in urgent events.

Reports of the Expert Group on Melamine Incident

1. First Report of the Expert Group on Melamine Incident. 20 October 2008. http://www.legco.gov.hk/yr08-09/english/panels/hs/papers/hscb2-101-1-e.pdf
2. Second Report of the Expert Group on Melamine Incident. 21 January 2009. http://www.fhb.gov.hk/melamine/doc/e_report20090121.pdf
3. Third Report of the Expert Group on Melamine Incident. 15 April 2009. http://www.fhb.gov.hk/download/press_and_publications/otherinfo/081020_melamine/e_report20090415.pdf

References

1. Chen JS. A worldwide food safety concern in 2008—melamine-contaminated infant formula in China caused urinary tract stone in 290,000 children in China. Chin Med J (Engl) 2009;122:243-4.
2. Wang Z, Luo H, Tu W, et al. Melamine-tainted milk product-associated urinary stones in children. Pediatr Int 2011;53:489-96.
3. Kong AP, Choi KC, Ho CS, et al. Hong Kong Chinese school children with elevated urine melamine levels: a prospective follow up study. BMC Public Health 2011;11:354.
4. Panesar NS, Chan KW, Lo WS, Leung VH, Ho CS. Co-contamination, but not mammalian cell conversion of melamine to cyanuric acid the likely cause of melamine-cyanurate nephrolithiasis. Clin Chim Acta 2010;411:1830-1.
5. Chu CY, Chu KO, Chan JY, et al. Distribution of melamine in rat foetuses and neonates. Toxicol Lett 2010;199:398-402.
6. Chan JY, Lau CM, Ting TL, et al. Gestational and lactational transfer of melamine following gavage administration of a single dose to rats. Food Chem Toxicol 2011;49:1544-8.
7. Chu CY, Chu KO, Ho CS, et al. Melamine in prenatal and postnatal organs in rats. Reprod Toxicol 2013;35:40-7.
8. Partanen H, Vähäkangas K, Woo CS, et al. Transplacental transfer of melamine. Placenta 2012;33:60-6.
9. Peng J, Li D, Chan YK, et al. Effects of water uptake on melamine renal stone formation in mice. Nephrol Dial Transplant 2012;27:2225-31.
10. Poon NW, Gohel MD, Lau C, Hon EK, Leung PC, Ng CF. Melamine crystallization: physicochemical properties, interactions with other lithogenic salts and response to therapeutic agents. J Urol 2012;187:1483-90.
11. Wu XW, Lee CC, Muzny DM, Caskey CT. Urate oxidase: primary structure and evolutionary implications. Proc Natl Acad Sci U S A 1989;86:9412-6.
12. Yazdi SH, White IM. Optofluidic surface enhanced Raman spectroscopy microsystem for sensitive and repeatable on-site detection of chemical contaminants. Anal Chem 2012;84:7992-8.
13. Hsieh YT, Chen WT, Tomalová I, Preisler J, Chang HT. Detection of melamine in infant formula and grain powder by surface-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 2012;26:1393-8.
14. Zhang Z, Cooks RG, Ouyang Z. Paper spray: a simple and efficient means of analysis of different contaminants in foodstuffs. Analyst 2012;137:2556-8.
15. Zhang Q, Gamboa da Costa G, Von Tungeln LS, Jacob CC, Brown RP, Goering PL. Urinary biomarker detection of melamine- and cyanuric acid-induced kidney injury in rats. Toxicol Sci 2012;129:1-8.
16. Kim TH, Ahn MY, Lim HJ, et al. Evaluation of metabolomic profiling against renal toxicity in Sprague-Dawley rats treated with melamine and cyanuric acid. Arch Toxicol 2012;86:1885-97.
17. Schnackenberg LK, Sun J, Pence LM, Bhattacharyya S, Gamboa da Costa G, Beger RD. Metabolomics evaluation of hydroxyproline as a potential marker of melamine and cyanuric acid nephrotoxicity in male and female Fischer F344 rats. Food Chem Toxicol 2012;50:3978-83.
18. Yang Y, Xiong GJ, Yu DF, et al. Acute low-dose melamine affects hippocampal synaptic plasticity and behavior in rats. Toxicol Lett 2012;214:63-8.
19. An L, Li Z, Yang Z, Zhang T. Melamine induced cognitive impairment associated with oxidative damage in rat's hippocampus. Pharmacol Biochem Behav 2012;102:196-202.
20. World Health Organization. Toxicological and health aspects of melamine and cyanuric acid. Geneva, World Health Organization 2009. Available at: http://www.who.int/foodsafety/publications/chem/ Melamine_report09.pdf.

21. Expert Group on Melamine Incident. First Report of the Expert Group on Melamine Incident. 20 October 2008. Available at: http://www.fhb.gov.hk/melamine/doc/report20081020.pdf.
22. Lam HS, Ng PC, Chu WC, et al. Renal screening in children after exposure to low dose melamine in Hong Kong: cross sectional study. BMJ 2008;337:a2991.
23. Lui SF. Melamine tainted milk products—situation in Hong Kong. Hong Kong Med Diary 2008;13:31-2.

Two-year follow-up for children with melamine exposure in Hong Kong: a multicentre study

HSHS Lam 林鴻生
WM Lai 賴偉明
SN Wong 黃錫年
MC Chiu 趙孟準
WCW Chu 朱昭穎
PC Ng 伍百祥
MHM Chan 陳浩明
CWK Lam 林偉基
NKC Tse 謝紀超
NS Kwong 鄺毅山
TWL Mak 麥永禮

Key Messages

1. The level of melamine exposure in Hong Kong children was near the tolerable daily limit set by the World Health Organization.
2. No evidence of renal dysfunction was noted in subjects over the 2-year follow-up period.
3. There was no evidence that low levels of melamine exposure were associated with haematuria, proteinuria, or ultrasound abnormalities.
4. Asymptomatic children (with no evidence of acute renal complications) after low-dose melamine exposure do not need routine follow-up.

Hong Kong Med J 2013;19(Suppl 8):S10-11

Department of Paediatrics, The Chinese University of Hong Kong
HSHS Lam, PC Ng
Department of Paediatrics and Adolescent Medicine, Princess Margaret Hospital
WM Lai, MC Chiu, NKC Tse
Department of Paediatrics and Adolescent Medicine, Tuen Mun Hospital
SN Wong, NS Kwong
Department of Imaging and Interventional Radiology, The Chinese University of Hong Kong
WCW Chu
Department of Chemical Pathology, The Chinese University of Hong Kong
MHM Chan, CWK Lam
Toxicology Reference Laboratory, Princess Margaret Hospital
TWL Mak

Principal applicant and corresponding author:
Dr Hugh Simon Hung San Lam
Department of Paediatrics, The Chinese University of Hong Kong, 6/F Clinical Sciences Building, Prince of Wales Hospital, Shatin, NT, Hong Kong SAR, China
Tel: (852) 2632 2851
Fax: (852) 2636 0020
Email: hshslam@cuhk.edu.hk

背景：在二零零八年，部份中國大陸的兒童被發現其急性腎臟症狀與攝取高劑量三聚氰胺有關。而香港兒童的三聚氰胺攝取量則接近世界衛生組織所訂的每日可容忍攝入量（按每公斤體重計算為0.2毫克）。因此本研究收集關於攝取低劑量三聚氰胺後對兒童健康影響的數據。
目標：調查香港兒童攝取三聚氰胺後其腎臟的中長期影響。
研究方法：在香港健康中心進行檢查的兒童如被發現有以下任何一項情況會被跟進：（1）超聲波顯示有腎結石，沈積物或阻塞物，（2）尿液試紙檢測結果異常。合適的兒童會根據以下條件決定召募的優先次序：（1）三聚氰胺估計攝取值，（2）腎病的臨床徵狀，（3）年紀較輕。所有參與者均接受了腎臟連續超聲波掃瞄，尿液檢測，尿液中Beta2微球蛋白含量測試及肌酸酐清除試驗（從單次血清肌酐酸濃度中計算）。另外，超聲波掃瞄顯示異常的兒童會接受進一步檢查以找出形成腎結石的原因。
結果：研究包括了62名在超聲波檢測發現異常及321名尿液樣本發現異常的兒童。攝取超過世界衛生組織所訂的每日可容忍攝入量（0.2微克/公斤體重）的兒童與並無超標的兒童之間並無發現臨床上的顯著差異。所有參與的兒童的腎功能參量亦處於正常水平。低三聚氰胺劑量估計攝取值與腎臟的症狀並無關係。
結論：研究並未發現三聚氰胺的估計攝取值與中長期腎臟不良後果有關。
建議：無證據支持需要對曾攝取低劑量三聚氰胺但無症狀的兒童進行定期跟進。

Introduction

In 2008, an outbreak of acute renal problems among children in the Chinese Mainland was linked to ingestion of melamine-tainted milk products (MTMPs).[1,2] In animal studies, the main adverse effects of melamine exposure include renal stones, renal tubular necrosis, melamine crystalluria, and haematuria.[3] There was no such study on humans. In Hong Kong, a large scale screening programme was initiated by the government.[1] Many asymptomatic children who were exposed to MTMPs were found to have abnormal urinalysis or ultrasound findings. The clinical significance of the renal stones and deposits, and asymptomatic haematuria was unknown. This study aimed to investigate whether Hong Kong children with low melamine exposure have adverse renal outcomes.

Methods

This study was conducted from April 2009 to September 2011. Children with abnormal urinalysis or ultrasound findings were recruited from three centres; priority was given to those estimated to have been exposed to melamine, had clinical features of renal disease or were of a young age. Recruited subjects were followed up and received repeated renal ultrasound scans and urine testing for renal function over a 2-year period. Ethical approval was obtained from the relevant Cluster Clinical Research Ethics Committees. Written informed parental consent was obtained from all cases.

Results

We recruited 62 children with ultrasound abnormalities and 321 with urine test abnormalities. Ultrasound abnormalities included renal stones, echogenic foci compatible with renal deposits, and a dilated renal pelvis. None of the children developed renal failure or life-threatening complications. In some children, ultrasound abnormalities resolved without treatment. Urine test abnormalities

included blood and/or protein positive on urine reagent strip testing. Only 3.4% of children were confirmed to have haematuria on microscopy. Among children who had marginally raised early morning urine protein/creatinine ratio, no other results were abnormal and they were referred to specialist renal clinics for further follow-up. Renal function estimates for all children were well within normal limits throughout the study period. None of the markers of renal outcomes measured deteriorated over the course of the 2-year follow-up.

Discussion

After 2 years of follow-up, no renal function abnormalities were detected in our subjects. The prevalence of haematuria and proteinuria was 0.2% each. There was no evidence that low-dose melamine exposure increases the risk of haematuria, proteinuria or renal dysfunction. Most abnormalities detected were unrelated to melamine consumption. No children had renal function deterioration over the 2-year follow-up. According to studies from the Chinese Mainland, although patients had acute problems, there were no long-term adverse effects.[4,5]

The main limitation of our study was that not all children who were screened were followed up. Nonetheless, the selection criteria prioritised children with highest risk of developing adverse renal complications. We were therefore able to undertake a relatively long period of follow-up to measure several markers of renal function and tubular damage on a cohort of children. Furthermore, despite involving only three centres, we assessed 55% of all children screened in the 2008 screening programme. This should provide a reasonable representation of the population. The second limitation was the lack of an objective marker of melamine exposure. However, detailed food questionnaires enabled a reasonable estimate of melamine exposure.

There was no evidence to support routine follow-up of asymptomatic children with no acute renal complications of melamine exposure. Low levels of melamine exposure are unlikely to pose a long-term health risk.

Acknowledgements

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-FU-01). The investigators would also like to acknowledge the contributions of the following people: Dr Cherry Ng, Dr Cheung Hon Ming, Dr William Wong, Mr Victor Kwok Ka Ming, and Miss Stephanie Chau Chi Lok. We also thank the subjects and their parents for their participation.

References

1. Lam HS, Ng PC, Chu WC, et al. Renal screening in children after exposure to low dose melamine in Hong Kong: cross sectional study. BMJ 2008;337:a2991.
2. Chiu MC. Melamine-tainted milk product (MTMP) renal stone outbreak in humans. Hong Kong Med J 2008;14:424-6.
3. Cianciolo RE, Bischoff K, Ebel JG, Van Winkle TJ, Goldstein RE, Serfilippi LM. Clinicopathologic, histologic, and toxicologic findings in 70 cats inadvertently exposed to pet food contaminated with melamine and cyanuric acid. J Am Vet Med Assoc 2008;233:729-37.
4. Shen Y, Sun Q, Gao J, et al. One year follow up of the outcomes of child patients with melamine-related kidney stones in Beijing and surrounding provinces in China. Nephrology (Carlton) 2011;16:433-9.
5. Sun N, Shen Y, He LJ. Histopathological features of the kidney after acute renal failure from melamine. N Engl J Med 2010;362:662-4.

CK Wong 黃振國
MHM Chan 陳浩明
JSS Kwok 郭崇聖
CS Ho 何宗信
PC Ng 伍百祥
SH Suen 孫錫鴻
KP Fung 馮國培
CM Lau 劉秋銘
TF Fok 霍泰輝

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.[1]

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

Hong Kong Med J 2013;19(Suppl 8):S12-5

The Chinese University of Hong Kong:
Department of Chemical Pathology
CK Wong, MHM Chan, JSS Kwok, CS Ho
Department of Paediatrics
PC Ng
Department of Obstetrics and Gynaecology
SH Suen
School of Biomedical Sciences
KP Fung
Faculty of Medicine
TF Fok
Government Laboratory, Hong Kong
CM Lau

Principal applicant and corresponding author:
Dr Chun Kwok Wong
Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, NT, Hong Kong SAR, China
Tel; (852) 2632 3338
Fax: (852) 2636 5090
Email: ck-wong@cuhk.edu.hk

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.[2] 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.[3] 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.[4,5] 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.[4,5] 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 pre-natal 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 (μg/kg/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 μg/kg/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) μg/kg/day. All cases had a daily exposure lower than the World Health Organization tolerable daily intake of 200 μg/kg/day. Only one subject had a daily exposure higher than the US Food and Drug Administration tolerable daily intake of 63 μg/kg/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) μg/mL. The reference interval established by the non-parametric percentile method was ≤35.8 μg/mL (95th percentile) [90% confidence interval, 23.6-58.7 μg/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 (μg/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

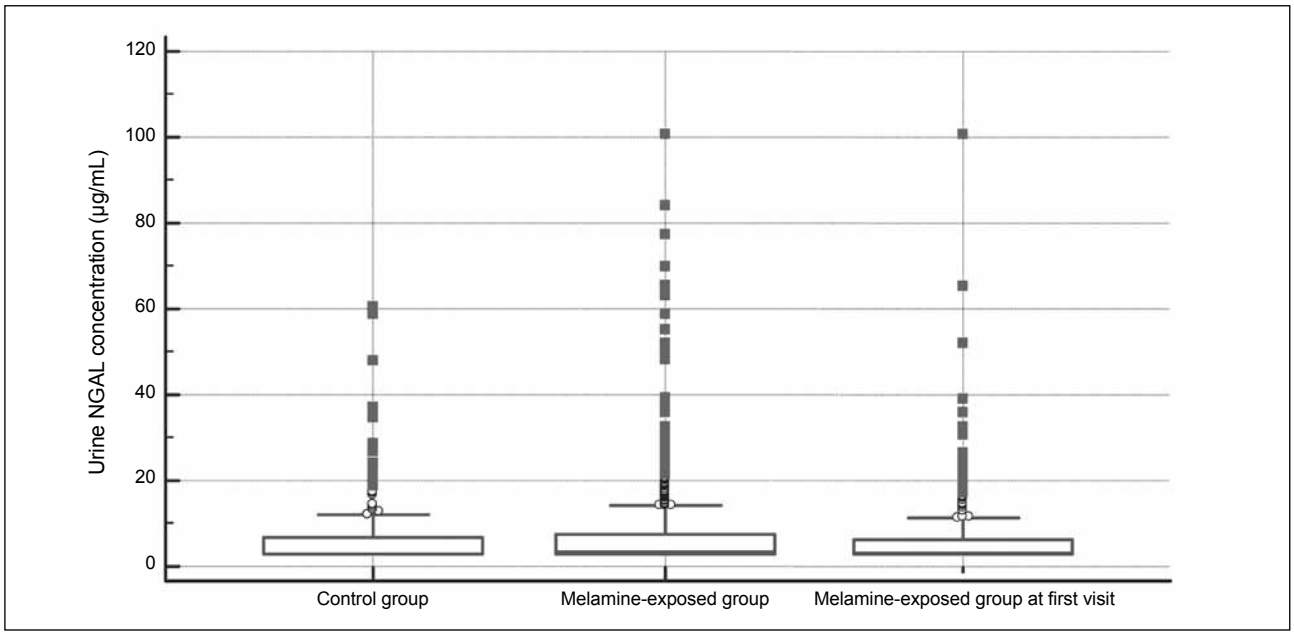

Fig. Box-and-Whisker plot of urine neutrophil gelatinase-associated lipocalin (NGAL) concentrations in the melamine-exposed (including first-visit sample) and control groups

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.

Acknowledgements

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-LAB-02). We thank all parents and children who participated in the study. We thank Dr Lam Hugh Simon Hung San and his clinical colleagues for organising sample collection. We also thank Ms Wong Tsz Yan and Ms Lo Wai Shan for performing the laboratory analyses.

References

1. World Health Organization. Toxicological and health aspects of melamine and cyanuric acid: report of a WHO expert meeting in collaboration with FAO, supported by Health Canada, Ottawa, Canada, 1-4 December 2008.
2. Kong AP, Choi KC, Ho CS, et al. Hong Kong Chinese school children with elevated urine melamine levels: a prospective follow up study. BMC Public Health 2011;11:354.
3. Panesar NS, Chan KW, Lo WS, Leung VH, Ho CS. Co-contamination, but not mammalian cell conversion of melamine to cyanuric acid the likely cause of melamine-cyanurate nephrolithiasis. Clin Chim Acta 2010;411:1830-1.
4. Chu CY, Chu KO, Chan JY, et al. Distribution of melamine in rat foetuses and neonates. Toxicol Lett 2010;199:398-402.
5. Chan JY, Lau CM, Ting TL, et al. Gestational and lactational transfer of melamine following gavage administration of a single dose to rats. Food Chem Toxicol 2011;49:1544-8.

APS Kong 江碧珊
XL Yang 楊酉林
JCN Chan 陳重娥
CWK Lam 林偉基
W Chu 朱昭穎
KC Choi 蔡繼洲

Prevalence of melamine exposure in Hong Kong children

在這項研究中，我們共化驗了502個尿液樣本。這些尿液樣本是從一項於2007-08年度進行的全港性系統學童評估中收集而來的。該項評估共有2119名香港華裔學童參與，他們是隨機從5間小學（804人）和6間中學（1315人）中邀請參加的。基於已發表的文獻，我們定義尿液三聚氰胺肌酸酐比例高於7.1 μg/mmol為高尿液三聚氰胺水平。我們聯絡及邀請被檢測有高尿液三聚氰胺水平之學童於2009年回來作臨牀評估（包括尿液分析及超聲波檢查）。這項研究共發現9%（47/502人）學童有高尿液三聚氰胺水平。一名學童拒絕回來檢查。46名回來檢查的學童中（平均±標準偏差年齡為13.9±2.9歲；28%男生），所有學童的泌尿系統超聲波檢查都是正常的。他們的奶類食品使用記錄跟尿液中三聚氰胺水平也沒有任何相關係。基於這項短期跟蹤研究（中位數為23.5月），香港華裔學童被檢測有高尿液三聚氰胺水平都健康良好，並沒有不良或嚴重的後遺症。

Key Messages

1. About 9% of Hong Kong school children had elevated urine melamine/creatinine ratios of >7.1 μg/mmol.
2. There was no association between milk consumption and urinary melamine levels.
3. Hong Kong school children with high urine melamine levels appeared to have benign clinical course.

Introduction

In early September 2008, melamine-tainted milk products in Mainland China raised public concerns about food safety.[1] Melamine is widely used in plastics, dishware, laminates, glues, and toy coatings. It is a potential food contaminant and a public health hazard. Excessive melamine exposure has been sporadically reported in Hong Kong. The incidence of renal involvement (echogenic renal foci) has been reported to be 0.03% to 0.6%, depending on selection criteria and evaluation methods.[2-4] From 2007 to 2008, we conducted a territory-wide survey to examine the prevalence of this metabolic syndrome in Hong Kong youths. Using archived urine samples, we assayed the urine melamine levels in a sub-cohort and evaluated their clinical status after 2 years. We also determined the correlation between urinary melamine levels and daily milk consumption.

Methods

This study was conducted from April 2009 to March 2011. Stored urine aliquots were collected from a territory-wide cohort surveyed in 2007 to 2008. A total of 2119 Hong Kong Chinese school children (67% girls) aged 6 to 20 years from five primary schools (804 children) and six secondary schools (1315 children) were randomly selected using a cluster sampling method. Informed consents were obtained from both the participants and their parents or guardians. The study was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. In a validated one-minute dietary questionnaire, participants' habit of milk consumption was inquired using a question "Are you drinking more than one cup of milk per day?" Spot morning urine specimens were collected from all agreed participants during the field study for measurement of the albumin/creatinine ratio (ACR). Albuminuria was defined as an ACR of >3.5 μg/mmol. A total of 502 urine aliquots were assayed for melamine level. High urine melamine level was defined as a urine melamine/creatinine ratio of >7.1 μg/mmol.[5] Subjects with high urine melamine level were invited for clinical evaluation in 2009 including urinalysis and ultrasound imaging of the urinary system. Renal ultrasonography was performed by an experienced radiographer using the Philips ATL HDI5000 ultrasound machine. Both kidneys were evaluated for the presence of renal stones, hydronephrosis or related renal scarring.

Urine melamine was measured by a liquid chromatography tandem mass spectrometry method. An aliquot of 20 μL of urine was added to 200 μL acetonitrile (ACN) containing 10 ppb stable isotope labelled melamine

Hong Kong Med J 2013;19(Suppl 8):S16-9

The Chinese University of Hong Kong:
Department of Medicine and Therapeutics
APS Kong, XL Yang, JCN Chan
Department of Chemical Pathology
CWK Lam
Department of Diagnostic Radiology and Organ Imaging
W Chu
Nethersole School of Nursing
KC Choi

Principal applicant and corresponding author:
Prof Alice Pik Shan Kong
Department of Medicine and Therapeutics, The Chinese University of Hong Kong, 9/F, Prince of Wales Hospital, Shatin, NT, Hong Kong SAR, China
Tel: (852) 2632 1558 / 2632 3307
Fax: (852) 2637 3852
Email: alicekong@cuhk.edu.hk

internal standard ($^{13}C_3$, $^{15}N_3$-melamine, Cambridge Isotope Laboratories, Andover, MA, USA). The solution was vortex mixed before centrifugation at 16 000 g for 5 minutes and the clear supernatant was transferred to a sample vial. Measurement was performed on a UPLC Waters Xevo TQ System (Waters, Milford, MA, USA). Calibrators (10-1000 μg/L) were prepared by spiking appropriate amounts of melamine into a negative pooled urine sample. An aliquot of 5 μL of calibrators/extracted melamine was injected into an ACQUITY UPLC BEH HILIC column (2.1x150 mm, 1.7 μm), which was kept at 45ºC. Weak and strong wash solutions for UPLC were ACN and water, respectively. We used 50% ACN in water as the seal wash solution. Melamine and the internal standard were separated from matrix interference by a gradient programme using mobile-phase solutions of 10 mM ammonium acetate in water and 10 mM ammonium acetate in 97% ACN in water at a flow rate of 500 μL/min. For the mass analyser, capillary voltage was optimised at 3.9 KV, cone voltage at 40 V, and collision energy at 24 V. The source and desolvation temperatures were at 150ºC and 500ºC, respectively. Positive electrospray ionisation tandem MS analyses were performed using *m/z* of 127 to 185 and 127 to 168 as quantitative and qualitative MRMs for melamine, respectively; and *m/z* of 133 to 189 as the MRM for the internal standard. The dwell time for each MRM was 50 msec. Both melamine and the internal standard eluted at around 3.5 minutes. Additional mobile-phase gradient programming was used to remove matrix interference and recondition the column for the next analysis. Injection-to-injection time was 6 minutes. Quantitation was performed by the TargetLynx Manager of the Waters MassLynx 4.1 software. The limit of quantitation was 5 μg/L and the linearity was up to 10 000 μg/L. Between-batch precision coefficients of variation for quality control samples (10, 100, 400 and 1000 μg/L) were <10%. Recoveries for spiked standards into blank matrix at concentrations of 100, 400, and 1000 μg/L were >99%.

Urine albumin and creatinine were measured on a Roche Modular Analytics system (Roche Diagnostics GmbH, Mannheim, Germany). Urine albumin was measured by turbidimetry and urine creatinine by the kinetic Jaffe reaction using standard reagent kits provided by the instrument manufacturer. Their analytical performances were within the manufacturer's specifications.

Urinalysis was performed by Multistix (Siemens urine test strips 10SG, Bayer). Microscopic examination was performed, by examining 60 μL of urine in microtitre plates using an inverted microscope, to look for red blood cells, casts, and crystals in urine. Quantitative culture of urine was performed on a chromogenic medium (CPS ID3 [Biomerieus] plate using 10 μL standard loops incubated aerobically for 18 to 24 hours at 35ºC.

Baseline characteristics in subjects with and without high urinary melamine levels (urine melamine/creatinine ratio, >7.1 μg/mmol) were compared using the Pearson's Chi square test, Fisher's exact test, T-test, and Mann-Whitney *U* test, as appropriate. Association between daily milk consumption and melamine level was assessed using Mann-Whitney *U* test and Pearson's Chi-square test, depending on the data format of the melamine level. The Spearman correlation coefficient was used to assess the correlation between urine ACR and the urine melamine/creatinine ratio. All statistical tests were two-sided, and a P value <0.05 was considered statistically significant.

Results

Table 1 shows the baseline characteristics of the 502 school children recruited in 2007 to 2008. The median spot urine melamine/creatinine ratio was 0.8 (range, undetectable to 1467) μg/mmol; in 213 (42%) the ratio was undetectable (zero). Urine albumin ranged from undetectable to 207 μg/mmol; the median urine ACR was 0.70 (interquartile range, 0.45-2.01) μg/mmol. Age, body weight, body height, and body mass index were associated with elevated urinary melamine levels (Table 1). There was no significant correlation between the urine melamine/creatinine ratio and ACR. There were 25 subjects with missing milk consumption data. In the 477 subjects with available data, no significant correlation was noted between the milk consumption and urine analysis (Table 2).

Of 47 (9%) subjects with high urine melamine levels, 46 (28% boys; mean±standard deviation age, 13.9±2.9 years) were followed up in 2009 (Table 3). The median follow-up duration was 23.5 (interquartile range, 19.8-30.6) months. None had any abnormality in the urinary system based on ultrasonography. None recalled any significant urinary tract symptoms or had abnormalities on urinalysis.

Discussion

In agreement with a previous report of children aged ≤12 years who consumed milk products contaminated with melamine,[2] this study also did not detect any major adverse renal outcomes in older school children with elevated urine melamine level. There was no association between the urine albumin level and the melamine level in subjects with high urine melamine levels.

In this cohort, young age, low body weight and height, low body mass index were all associated with elevated urinary melamine level. As younger school children tend to have higher milk consumption, age may be a factor linking urinary melamine level and anthropometric parameters, although there was no significant correlation between milk consumption and urinary melamine level, probably due to the small sample size. In young children who were not breast-fed, milk products, particularly the powdered infant formula, are the major sources of nutrients in infants. In 2008, the discovery of melamine contamination of these milk products in Mainland China raised alarm in the

Table 1. Baseline characteristics of the study sample (n=502) in 2007 to 2008

Characteristics	All (n=502)	Urine melamine/creatinine ratio of >7.1 μg/mmol		P value (test)
		No (n=455)	Yes (n=47)	
No. (%) of males	167 (33.3)	153 (33.6)	14 (29.8)	0.595 (Chi square test)
No. (%) of females	335 (66.7)	302 (66.4)	33 (70.2)	
Mean±SD age (years)	13.2±3.0	13.3±3.0	12.0±2.8	0.004 (T-test)
Mean±SD body weight (kg)	43.6±11.8	44.1±11.7	39.3±12.1	0.008 (T-test)*
Mean±SD body height (cm)	152.3±13.6	152.8±13.4	147.4±14.6	0.010 (T-test)*
Mean±SD body mass index (kg/m^2)	18.5±3.0	18.6±3.0	17.5±2.4	0.026 (T-test)*
Weight status (No. [%] of participants)				0.193 (Fisher's exact test)
Normal	428 (85.3)	384 (84.4)	44 (93.6)	
Overweight	51 (10.2)	48 (10.5)	3 (6.4)	
Obesity	23 (4.6)	23 (5.1)	0	
Median (IQR) urine albumin/creatinine ratio (μg/mmol)	0.70 (0.45-2.01)	0.69 (0.44-3.23)	0.70 (0.50-1.39)	0.820 (non-parametric Mann-Whitney *U* test)
Median (IQR) urine melamine/creatinine ratio (μg/mmol)	0.76 (0-2.62)	0.52 (0-1.73)	13.21 (9.09-21.55)	<0.001 (non-parametric Mann-Whitney *U* test)

* Not significant difference after adjusting for age

Table 2. Association between urine melamine level and daily milk consumption

Parameter	Drinking more than a cup of milk per day*		P value (test)
	No (n=378)	Yes (n=99)	
Median (IQR) urine melamine (μg/L)	6.0 (0-25.0)	11.0 (0-33.0)	0.280 (Mann-Whitney *U* test)
Median (IQR) urine melamine/creatinine ratio (μg/mmol)	0.62 (0-2.33)	1.00 (0-3.19)	0.182 (Mann-Whitney *U* test)
Urine melamine/creatinine ratio of >7.1 μg/mmol (No. [%] of subjects)			0.072 (Chi square test)
No	347 (91.8)	85 (85.9)	
Yes	31 (8.2)	14 (14.1)	

* 25 subjects with missing milk-drinking data

Table 3. Results of 46 subjects with urine melamine/creatinine ratio of >7.1 μg/mmol in 2009

Parameter	Result
No. (%) of males	13 (28.3)
No. (%) of females	33 (71.7)
Mean±SD age (years)	13.9±2.9
Mean±SD body weight (kg)	47.9±15.3
Mean±SD body height (cm)	155.9±11.8
Mean±SD body mass index (kg/m^2)	19.7±4.5
Urine total protein (g/L) [No. (%) of subjects]	
<0.1	35 (77.8)
0.1-0.2	6 (13.3)
0.2-0.3	2 (4.4)
0.3-0.4	0
≥0.4	2 (4.4)
Median (IQR) urine albumin/creatinine ratio (μg/mmol)	0.70 (0-2.55)
No. (%) of abnormal ultrasound finding on the urinary system	0

international community. According to the Ministry of Health of China, since 21 September 2008, almost 40 000 children had consumed melamine-tainted milk, with 13 000 hospitalisations and acute renal failure in 104 children.[1] More than 52 000 children and infants had sought medical treatment in Mainland China, with four reported deaths.

As melamine is not metabolised and rapidly eliminated in the urine of cats and dogs, melamine and its structural analogues, such as cyanuric acid, may interact and form melamine-cyanurate crystals. Human and primates have much higher uric acid concentrations in the blood, and melamine-urate crystals are more likely to form. Animals fed with melamine developed kidney stones causing urinary tract obstruction. Deaths secondary to urinary tract stones and acute renal failure in infants and young children exposed to very high melamine levels for prolonged period have been reported. Melamine is also widely used in plastics, dishware, adhesives, and toy coatings. Thus, ingestion of melamine as environmental pollutants may be a silent health hazard in children.

In our study, selection bias was unlikely owing to the random nature of the school sampling and the territory-

wide survey having been conducted before the melamine-tainted formula became an issue. Thus, potential recall bias in reporting milk consumption was not likely and might well reflect the real picture of melamine ingestion from food and environmental pollution. In previous reports of renal incidents,[2-4] subjects with known consumption of melamine-contaminated milk products or referred from designated outpatient clinics to special assessment centres were studied. These subjects might have had symptoms or their parents might have had increased alertness to seek medical attention. Our study had the advantage of being capable of identifying subclinical/silent cases missed in previous studies.

There are several limitations in our study. First, only one random spot urine specimen was collected. School children with high melamine levels might have transient exposure to melamine-tainted food products. Second, whether prolonged or repeated exposure to consumption of melamine-contaminated food might lead to long-term adverse renal outcome cannot be addressed by this short-term study. Third, the sample size was relatively small (502 children). Fourth, a detailed food diary was not recorded when the urine was sampled in 2007 to 2008.

Conclusions

About 9% of the study cohort had elevated urinary melamine level. There was no association between milk consumption and urinary melamine levels. In this short-term follow-up study, Hong Kong Chinese school children with high urine melamine levels appeared to have a benign clinical course. Longer follow-up is required to detect any long-term adverse clinical effects.

Acknowledgements

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-FU-04). We thank all school personnel, parents, and participants. Special thanks are extended to Dr Eric Liu, Ms Edith Kwok, and all research nurses and assistants for conducting this study.

Results of this study were published in: Kong AP, Choi KC, Ho CS, et al. Hong Kong Chinese school children with elevated urine melamine levels: a prospective follow up study. BMC Public Health 2011;11:354.

References

1. Parry J. China's tainted infant formula sickens nearly 13,000 babies. BMJ 2008;337:a1802.
2. Lam HS, Ng PC, Chu WC, et al. Renal screening in children after exposure to low dose melamine in Hong Kong: cross sectional study. BMJ 2008;337:a2991.
3. Ho SS, Chu WC, Wong KT, et al. Ultrasonographic evaluation of melamine-exposed children in Hong Kong. N Engl J Med 2009;360:1156-7.
4. Lau HY, Wong CS, Ma JK, Kan E, Siu KL. US findings of melamine-related renal disorders in Hong Kong children. Pediatr Radiol 2009;39:1188-93.
5. Lam CW, Lan L, Che X, et al. Diagnosis and spectrum of melamine-related renal disease: plausible mechanism of stone formation in humans. Clin Chim Acta 2009;402:150-5.

Melamine toxicity in rat foetuses and infants

CC Wang 黃志超
KP Fung 馮國培
TF Fok 霍泰輝
TK Lau 劉子健
CP Pang 彭智培
KO Chu 朱啟安
ASW Shum 沈秀媛
CWK Lam 林偉基
MHM Chan 陳浩明
CS Ho 何宗信
CM Lau 劉秋銘
TL Ting 丁大倫
TCW Mak 麥松威

背景：三聚氰胺在胎兒和幼兒的毒性不完全清楚。
主旨：研究三聚氰胺在大鼠胎兒和幼兒的藥物動力、效力及致畸作用。
方法和對象：三聚氰胺及其衍生物氰尿酸在大鼠胎兒和幼兒的毒性實驗。
實驗：妊娠、哺乳及產後大鼠給予三聚氰胺及氰尿酸，並取集胎兒和幼兒標本。
研究項目：三聚氰胺及氰尿酸在大鼠胎兒和幼兒的生物利用、母胎和母乳的轉遞及胎幼兒的分佈和積累、胚胎致畸、胎兒急性中毒及幼兒慢性中毒，以確定在發育及成長的安全性。
結果：三聚氰胺及氰尿酸可通過乳腺及胎盤從母親傳送到幼兒、胎兒及羊水。其動力學特性在妊娠期母體與產後幼兒相反。在妊娠末期胎兒腎臟及產後　周幼兒腎臟的生物利用度最高。胎兒半致死劑量為300毫克/千克，幼兒半中毒劑量為80毫克每千克，每日可容忍攝入量少於0.02毫克/千克。母親攝取三聚氰胺可引起體重及產子量降低，流產及圍產期亡率升高，但不會致畸。
結論：本實驗確定三聚氰胺及氰尿酸經母胎與母乳傳送，主要在未成熟的胎兒及幼兒腎臟分佈和積累。母親接觸可引起不良妊娠和圍產後果。
影響：本研究明確三聚氰胺在大鼠胎兒和幼兒的毒性，並提供有用的產前和產後藥理和毒理數據。

Key Messages

1. Melamine and cyanuric acid can transfer to mammary glands and pass through the placenta to foetuses and amniotic fluid.
2. In rats, pharmacokinetic profiles of maternal samples during gestation and of infant samples during the postnatal period were reversed.
3. High bioavailability was identified in foetal kidneys at late gestation and in infant kidneys at early postnatal period.
4. The foetal LD_{50} was defined as 300 mg/kg, whereas infant IC_{50} as <80 mg/kg and the tolerable daily intake as <0.02 mg/kg.
5. Maternal exposure resulted in no congenital malformation but decreased maternal weight gain and litter size as well as increased pregnancy loss and perinatal deaths.

Hong Kong Med J 2013;19(Suppl 8):S20-2

The Chinese University of Hong Kong:
Department of Obstetrics and Gynaecology
CC Wang, TK Lau, KO Chu
School of Biomedical Sciences
KP Fung, ASW Shum
Department of Paediatrics
TF Fok
Department of Ophthalmology and Visual Sciences
CP Pang, KO Chu
Department of Chemical Pathology
CWK Lam, MHM Chan, CS Ho
Department of Chemistry
TCW Mak
Government Laboratory, Hong Kong
CM Lau, TL Ting

Principal applicant and corresponding author:
Dr Chi Chiu Wang
Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, 1/F, Block E, Prince of Wales Hospital, Shatin, NT, Hong Kong SAR China
Tel: (852) 2632 2810
Fax: (852) 2636 0008
Email: ccwang@cuhk.edu.hk

Introduction

In 2004, melamine-tainted pet food resulted in numerous incidents of acute renal failure in dogs and cats.[1] In 2008, melamine-adulterated milk powder for infants was discovered in China.[2] The toxicity of melamine has raised concerns for public health in China and other countries. Safety data regarding melamine in pregnant and lactating women are limited. When melamine-contaminated food is consumed during pregnancy and lactation, foetuses and infants may become sensitive to the direct action or to environmental changes produced by chemicals or drugs,[3] and more vulnerable to the toxic effects of melamine. It is unknown whether high levels of melamine can pass through the placenta to the developing foetus, and whether melamine has any toxicity on embryo-foetal development and prenatal and postnatal growth. This study aimed to identify melamine and cyanuric acid toxicity in foetuses and infants during pregnancy and lactation. The specific objectives were to establish the pharmacokinetics, pharmacodynamics, and teratogenicity of melamine and cyanuric acid with respect to foetal and infant rats in vivo.

Methods

This study was conducted from April 2009 to March 2011. Sprague Dawley rats were used. According to the US Food and Drug Administration Guidelines on Detection of Toxicity to Reproduction for Medicinal Products, three experiments were performed to determine pharmacological profiles and potential effects of melamine and cyanuric acid on foetuses and infants. In part I, pharmacokinetics were studied with respect to the bio-availability of melamine and cyanuric acid by measuring their concentrations in foetal and infant samples. In part II, pharmacodynamics were studied with respect to the foetal and infant toxic dosage of melamine and cyanuric acid. In part III, teratogenic dose for potential developmental toxicity was determined based on congenital malformations following maternal exposure.

In part I, single bolus doses of melamine or cyanuric acid were administered to healthy female rats at different reproductive stages, including early, mid, and late pregnancy, and during lactation, as well as to healthy infants at different stages

of maturation. Samples were collected within 24 hours of administration to characterise pharmacokinetic profiles. In part II, single bolus doses of melamine and cyanuric acid in different concentrations were administered to healthy pregnant rats at different stages of pregnancy to determine acute toxicity to the foetus. In addition, multiple bolus doses of melamine and cyanuric acid in different concentrations were administered daily for 2 weeks to healthy infants at different stages of maturation. In part III, repeated bolus doses of melamine and cyanuric acid were administered daily to healthy pregnant rats (at defined gestational stages) and to lactating rats (at different developmental stages) to detect immediate and latent effects of such exposure.

Melamine and cyanuric acid were measured simultaneously by a liquid chromatography tandem mass spectrometry (LC-MS/MS) method as previously described.[4] The UPLC Waters Xevo TQ System (Waters, Milford, MA, USA) was used to detect the melamine and cyanuric acid accumulation in the samples as previously described.[5] Extraction of tissue homogenate samples was adapted using a previously reported method,[6] whereas extraction from serum and biological fluids entailed direct centrifugation at 16 000 g for 5 minutes. After extraction, clear supernatant was transferred to a new sample vial for direct LC-MS/MS analysis. Calibrator or extracted samples were injected into an ACQUITY UPLC BEH HILIC column. 10 ppb stable isotope-labelled melamine and cyanuric acid were added as internal standards ($^{13}C_3$,$^{15}N_3$-melamine/cyanuric acid; Cambridge Isotope Laboratories, Andover, MA, USA) to attain a 20 μL concentration in the combined aqueous layer. The limit of quantitation of both analyses was 5 ppb, and linearity was up to 10 000 ppb. Between-batch precision coefficients of variation for quality control samples (10, 100, 400, and 1000 mg/L) were <10%, and recoveries from spiked standards into the blank matrix were >99%. The detection limits were 0.005 ppm in both serum/biological fluids and in tissue homogenate.

General maternal effects on pregnant rats, and adverse effects on foetuses and infants were monitored. In part I, bioavailability of melamine and cyanuric acid in both maternal and foetal/infant samples were measured by analytical methods. The pharmacokinetic model and parameters of melamine and cyanuric acid during pregnancy and in foetuses and infants were determined. In part II, the effective foetal and infant toxicity doses of melamine and cyanuric acid were derived. In part III, adverse maternal and foetal outcomes were recorded to determine the developmental toxicity potential of melamine and cyanuric acid.

Results

Maternal and foetal/infant pharmacokinetics

During pregnancy, single dose maternal exposure to melamine and cyanuric acid at about 0.2 and 20 mg/kg had no adverse maternal or foetal outcomes. Pharmacokinetics of melamine and cyanuric acid in amniotic fluid and the whole foetus followed the one-compartment model, but in maternal serum and breast milk followed the non-compartmental model. The pharmacokinetic profiles of melamine and cyanuric acid were similar. Cmax, AUC, T½, and MRTinf in maternal serum were significantly decreased in early gestation and significantly increased in late gestation and after delivery. However, the parameters were reversed in breast milk, amniotic fluid, and whole foetus in late gestation. Tmax in amniotic fluid, breast milk, and the whole foetus were longer than that in maternal serum. Cmax in maternal serum was the highest among the samples. Cmax in breast milk, the whole foetus, and amniotic fluid were lower than in maternal serum. AUC in maternal serum was the largest among the samples. The longest T½ was in breast milk, followed by amniotic fluid, the whole foetus, and maternal serum.

In infant rats, single dose maternal exposure to melamine and cyanuric acid at about 0.2 and 20 mg/kg had no adverse postnatal outcomes. The pharmacokinetics of melamine in infant serum and organs followed the non-compartmental model. In all infant samples, Tmax, T, and Vz/F were significantly decreased, but Cmax and AUC were significantly increased in later postnatal periods. Tmax was greater in infant kidneys than in serum; Cmax and AUC in the infant kidneys were much lower than in serum; and T½ in serum was shorter than in the kidney. The pharmacokinetics of melamine and cyanuric acid in foetal organs followed the one-compartment model, but infant organs followed the non-compartment model. Highest bioavailability was identified in foetal kidneys at late gestation and in infant kidneys at the early postnatal period.

Foetal and infant toxicity

Foetal LD_{100} was determined as 2500 mg/kg and infant IC_{100} as 320 mg/kg. Adverse outcomes after exposure to melamine at higher concentrations were more severe and common in early gestation than exposure to cyanuric acid in late gestation. Significantly more adverse maternal and foetal outcomes were encountered in early gestation, including acute toxicity, miscarriage, growth restriction, abnormal placentation, foetal resorption, but no congenital malformation was identified. Adverse neonatal outcomes secondary to higher concentrations of melamine were more severe and common at the pre-weaning period than following cyanuric acid exposure in the later postnatal period. Adverse maternal and foetal outcomes were significantly more in early gestation, including acute toxicity, hypoglycaemia, abnormal liver/renal function, proteinuria, haematuria and renal stones/crystals. The foetal LD_{50} was estimated as 300 mg/kg, whereas infant IC_{50} as <80 mg/kg and the tolerable daily intake as <0.02 mg/kg.

Reproductive toxicity

Maternal exposure of melamine and cyanuric acid resulted in no congenital malformation but significantly decreased maternal weight gain and litter size as well as increased

pregnancy loss and perinatal death. The adverse pregnancy and perinatal outcomes after exposure to melamine at higher concentrations were more severe and common than exposure to cyanuric acid. No abnormal renal function or renal stone/crystals were identified in maternal and foetal kidneys at concentrations up to 50 mg/kg.

Discussion

High bioavailability of melamine can pass through the placenta and enter foetuses and amniotic fluid as well as mammary glands and breast milk. Melamine is a small and highly polar compound. In breast milk and foetuses, it has a longer Tmax owing to delayed tissue distribution and compartment effects in maternal rats and the exocrine process of mammary glands. Transfer of melamine through the placenta to the foetal circulation and tissues may contribute to the delay in Tmax. Melamine then circulates in the foetus and is distributed in various tissues and excreted by the kidney into the amniotic fluid leading to further delays. As a result, in amniotic fluid the Tmax of melamine was the longest.

Melamine has a half-life in plasma of about 3 hours. It is then excreted in urine as the original compound. In neonate kidney that has just developed, the half-life is longer. Comparing the λz, the ability to remove melamine from maternal serum during pregnancy and the neonatal serum was similar. This suggests that accumulation of melamine may occur in the neonatal kidney, where water is reabsorbed and urine is concentrated before excretion.

Although melamine is quickly removed by urine, sub-chronic and chronic administration of melamine in adults could increase the incidence of ulceration of the bladder epithelium, inflammation, and epithelial hyperplasia of the urinary bladder, as well as the cancer rate of the urinary bladder and ureter.[7] Although there is no evidence of any teratogenesis due to melamine, triethylene melamine exposure has been shown to cause foetal death prior to or around the time of implantation.[8] Our reproductive toxicity study in the embryo and foetal development confirmed that there were adverse pregnancy and perinatal outcomes, but no definite teratogenic effects.

The US Food and Drug Administration published an Interim Safety and Risk Assessment of Melamine and its Analogues in Food for Humans in 2008. The human tolerable daily intake of melamine is suggested to be 0.63 mg/kg/day (ppm/day), whereas the tolerable daily intake recommended by the European Food Safety Authority is 0.5 mg/kg/day. The World Health Organization adopted the tolerable daily intake to be 0.2 mg/kg/day, which is applicable to both adults and infants as an estimated maximum amount of daily melamine exposure over a lifetime. In the present study, foetal LD_{50} for acute cardiac toxicity in utero was about 300 mg/kg, whereas infant IC_{50} for chronic renal toxicity was <80 mg/kg and its tolerable daily intake was <0.02 mg/kg.

In conclusion, maternal melamine and cyanuric acid could pass through the placenta and enter foetuses and mammary glands. It could also be eliminated through the placenta of the foetuses and the kidneys of the neonates. Melamine and cyanuric acid accumulated mainly in foetal and infant kidneys during late gestation and early postnatal periods. Neither melamine nor cyanuric acid was teratogenic, but reproductive toxicity during pregnancy was confirmed. This study provided information on the potential short-, medium- and long-term developmental toxicity associated with foetal and infant exposure to melamine and cyanuric acid, as well as toxicity reference data to regulate the human products to fulfil safety for pregnant/lactating women and infants.

Acknowledgements

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-BS-06). We thank Judy Chan, XZ Liu, KS Law, Jasmine Lau, Tsui SK, WC Sham, Nina Chu, Priscilla Tang, H Xu and Lucia Li for their technical assistance and animal house at university campus and Prince of Wales Hospital for the animal care and supports.

References

1. Brown CA, Jeong KS, Poppenga RH, et al. Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. J Vet Diagn Invest 2007;19:525-31.
2. Parry J. China's tainted infant formula sickens nearly 13,000 babies. BMJ 2008;337:a1802.
3. Karnofsky DA. Drugs as teratogens in animals and man. Annu Rev Pharmacol 1965;10:447-72.
4. Heller DN, Nochetto CB. Simultaneous determination and confirmation of melamine and cyanuric acid in animal feed by zwitterionic hydrophilic interaction chromatography and tandem mass spectrometry. Rapid Commun Mass Spectrom 2008;22:3624-32.
5. Kong AP, Choi KC, Ho CS, et al. Hong Kong Chinese school children with elevated urine melamine levels: a prospective follow up study. BMC Public Health 2011;11:354.
6. Filigenzi MS, Tor ER, Poppenga RH, Aston LA, Puschner B. The determination of melamine in muscle tissue by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2007;21:4027-32.
7. Okumura M, Hasegawa R, Shirai T, Ito M, Yamada S, Fukushima S. Relationship between calculus formation and carcinogenesis in the urinary bladder of rats administered the non-genotoxic agents thymine or melamine. Carcinogenesis 1992;13:1043-5.
8. Generoso WM, Rutledge JC, Cain KT, Hughes LA, Downing DJ. Mutagen-induced fetal anomalies and death following treatment of females within hours after mating. Mutat Res 1988;199:175-81.

CF Ng 吳志輝
KLE Hon
MDI Gohel

Effects of melamine on urine crystallisation kinetics and cell responses

背景：在2007年年初，貓和狗急性腎功能衰竭的報告和假寵物食品有關。而在2008年9月中，中國也有兒童腎結石和/或腎功能衰竭的報告。這兩件事件都和三聚氰胺及其共同的污染物氰尿酸結石有關。
宗旨和目標：（1）在體外腎環境研究三聚氰胺的物理化學反應，（2）研究三聚氰胺是如何影響培養的腎細胞包括損傷，氧化應激，炎症反應和細胞基因表達。
研究的設計與方法：利用一個混合懸浮混合人工結晶去除器，研究在人體體外尿液中三聚氰胺的結晶。另外建立一個Transwell小細胞培養模型來研究三聚氰胺對腎細胞的影響。
結果：三聚氰胺晶體在人體尿液中的酸性條件（pH值5.0）下形式。三聚氰胺存在顯著增加其他結石鹽（尿酸、草酸鈣和磷酸鹽）的沉澱。臨床有關的藥物，如檸檬酸和碳酸氫鹽可以顯著減少三聚氰胺晶體的形成。在細胞培養研究中顯示，腎細胞是透過物理傷害產生氧化反應和細胞損傷（LDH釋放）。而細胞因子釋放和基因表達也顯示和炎症反應有關。
結論：三聚氰胺的晶體在酸性尿液中形式，三聚氰胺也可以增加其他結石鹽的結晶。檸檬酸和碳酸氫鈉治療是有效的。從細胞培養的研究顯示三聚氰胺透過物理損壞導致細胞和體液免疫反應。
啟示：三聚氰胺可能導致本身和其他鹽類（如尿酸和鈣有關的）晶體的形成。三聚氰胺當前的治療方案是有效的。三聚氰胺對細胞的長期影響似乎有限，因為三聚氰胺主要是透過物理方法引致細胞損傷和激起免疫細胞調動到損傷部位。

Key Messages

1. Melamine co-precipitates from human urine under acidic conditions and promotes precipitation of other lithogenic ions.
2. In vitro studies confirmed that citrate and bicarbonate therapy may help to inhibit melamine crystallisation.
3. Chinese herbal medicines had only acute efficacy (within 24 hours) after which they were not effective in lowering melamine crystallisation.
4. Cell culture studies demonstrated that the effect of melamine on cells differed from that of calcium oxalate crystals. Melamine crystals and aggregates caused physical damage to cells resulting in an inflammatory response.
5. Global gene expression microarray scanning revealed up-regulation of cytokines and down-regulation of calcium channels suggesting a cell protective mechanism.

Introduction

In 2008 in China, the presence of renal stones in infants and children was linked to intake of melamine-tainted milk. Six infants were reported to have died and over 300 000 suffered from kidney stones. In Hong Kong, 12 of 40 000 screened children were tested positive of kidney stones.[1] In a study of 3835 children attending Princess Margaret Hospital, 22 (0.6%) had renal disorders but not necessarily related to melamine.[2]

The physico-chemical properties of melamine in body fluids were largely unknown. In toxicological studies, melamine alone does not cause renal damage. However, when combined with cyanuric acid, insoluble crystals form and can obstruct renal tubules. Most of those affected were <3 years old. This study aimed to investigate melamine and cyanurate toxicity in terms of the physico-chemical interactions of melamine with human urine, and the process by which melamine affects renal cells in terms of deposit and uptake, transport, stress and inflammatory response, and gene expression.

Methods

This study was conducted from March 2009 to August 2010.

Urine crystallisation studies

Two parallel mixed-suspension-mixed-product removal (test and control) apparatuses were set up. These devices were water-jacketed (37ºC) with five openings, to allow for urine, melamine solution, cyanuric acid solution, and mixed product removal, as well as insertion of a probe for particle counting. The system was set up for melamine and cyanurate acid alone and then for different percentages of melamine:cyanurate acid (0-100%) and concentrations (0-10 mM). Crystallisation kinetic parameters such as particle nucleation rate, growth

Hong Kong Med J 2013;19(Suppl 8):S23-5

Department of Surgery, The Chinese University of Hong Kong
CF Ng
Department of Paediatrics, The Chinese University of Hong Kong
KLE Hon
Department of Health Technology and Informatics, The Hong Kong Polytechnic University
MDI Gohel

Principal applicant and corresponding author:
Prof Chi Fai Ng
Department of Surgery, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, NT, Hong Kong SAR, China
Tel: (852) 2632 2625
Fax: (852) 2637 7974
Email: ngcf@surgery.cuhk.edu.hk

rate, and particle suspension density were determined from the crystalliser through the Multisizer Particle Counter (Coulter).

Besides the effects of pH, minimum concentration, and ionic strength, we investigated the effects of (1) other lithogenic ions such as uric acid (UA), calcium oxalate (CaOx), and calcium phosphate (CaP), (2) clinically relevant therapeutic drugs such as potassium citrate and sodium bicarbonate, (3) Chinese herbal medicines (*Shi Wei* 石葦 and *Semen Coicis* 薏苡仁), and (4) urinary tract infection (UTI) or the presence of bacteria in the presence of melamine.

Cell culture studies with melamine and cyanurate acid

The presence of melamine and cyanurate acid in the lumen of the intestines and renal tubules prompted investigation of the interaction of both the soluble and crystallised forms in terms of (1) how they are absorbed and localised, and (2) how the crystal-cell interaction ensues.

Human (tubular, gastric) and canine (tubular) cell lines were used through a standard protocol in the transwell insert as described earlier for our CaOx studies.[3] This culture method separates the apical and basal portion of the cells and mimics the luminal environment. We investigated the concentrations of melamine, cyanurate, and melamine/cyanurate that caused cell toxicity. Cell growth was assessed using a Trypan blue-based ViCell counter. Apical and basal media were collected for measurement for cytokine panel expression using 6-plex and 11-plex assays. Oxidative DNA damage induced by melamine/cyanurate crystals was quantified using a highly sensitive 8-OHdG ELISA kit.

Results

Physicochemical aspects of melamine/cyanurate crystallisation

The use of the mixed-suspension-mixed-product removal (test and control) apparatus helped derive the nucleation rate (number of crystals/minute/mL), growth rate (μm/minute), and total particle suspension density (mmol). By studying various proportions of melamine:cyanurate, we found that at 1:1 ratio, crystallisation was maximal for the three outcome measures. The minimum concentration that would trigger crystallisation was 5 mM (at 1:1 ratio) and at 10 mM it was six-fold higher. A trend was observed that at higher pH values, there was less crystallisation. The maximal crystallisation for melamine was observed at pH 5.0. Since urine itself is supersaturated with other lithogenic ions, any change in ionic strength is subtle.

Melamine crystallisation with other endogenous urine factors

The effects of melamine/cyanurate on other lithogenic ions such as CaOx, UA and CaP were profound. As little as 0.1 mM of melamine and/or 5 mM cyanurate caused precipitation and significant changes in the nucleation rate and particle suspension density. Although higher concentrations (>5 mM) of cyanurate or the mixture were needed to cause precipitation of melamine, as little as ≤0.1 mM of melamine in urine would cause lithogenic salt precipitation.

Effects of therapeutic agents on melamine crystallisation

Current therapeutics for urolithiasis includes the use of drugs that chelates lithogenic ions to result in urine alkalisation or acidification. Two commonly used drugs, potassium citrate and sodium bicarbonate, and two popular Chinese herbal medicines, *Shi Wei* and *Semen Coicis* (Coix seeds), were studied. Increasing citrate significantly decreased the nucleation rate and particle suspension density, but had no effect on the growth rate; this effect was thought to be due to calcium chelation. For bicarbonate, increasing concentrations caused significant increases in the nucleation rate, with a decreased growth rate and particle suspension density. This suggests that the bicarbonate inhibited melamine crystallisation through reduced growth and particle density. Both drugs have been used clinically with good efficacy. Both Chinese herbal medicines significantly reduced the nucleation rate and particle suspension density. For *Shi Wei*, the diuretic action and reduced melamine crystallisation were sustained for up to 24 hours only.

Studies on cell culture with melamine and cyanurate crystallisation

To investigate whether the melamine/cyanurate crystals worked similarly, our initial cell culture studies were modelled on a CaOx transwell cell culture model.[3] No significant cytotoxic effect was observed in the gastric cells when the cells were incubated with different concentrations of melamine or cyanurate and their mixtures. Therefore, subsequent work was focused on renal cells.

Direct cytotoxic effects, as assessed by the release of LDH, were demonstrated by a mixture of melamine and cyanurate in a concentration-dependent manner, but not by melamine or cyanurate alone. On the monolayer (with tight junctions), about 15 to 25% of the cell viability was affected immediately after 10 minutes of agitation/incubation with a melamine/cyanurate mixture at 1:1 ratio, which was in contrast to the artificial urine control (irrespective of the concentrations added). We observed neither cell repair nor further reduction of viable cell numbers after 24 hours. In addition, there was no crystal adhesion on apical surface of cells, and no further cellular damage after prolonged (24 hours) incubation. The cytotoxic effects caused by melamine/cyanurate crystals at 1:1 and 99:1 ratios were due to physical contact during agitation applied at the initial stage of the experiment, and thus crystal uptake and endocytosis were not likely. Oxidative stress was significantly induced by melamine/cyanurate at 1:1 ratio, compared to the control ($P<0.01$).

Among the 16 Th1/Th2 cytokines and chemokines tested, baseline levels of IL-6, IL-8, and monocyte chemotactic protein were detected in the harvested media of the cultures at 24 hours, and higher levels were measured at the apical side. Secretions of IL-6, IL-8, and monocyte chemotactic protein were increased in parallel with their cytotoxic effects. Furthermore, IL-5 was not detected in the control media, whereas its secretion was also stimulated by the crystals. These findings suggested a shift of cell microenvironment towards a Th2 type (IL5 and IL6) immune response, which favours humoral responses. The overall microenvironment suggested that the crystals caused physical cell injury to the tubular cells, so as to trigger pro-inflammatory reactions.

Effect of melamine and cyanurate crystallisation on gene expression

Similar to a study on globally expressed genes during CaOx nephrolithiasis in rats, we used a gene expression microarray with whole genome scanning (Human genome 44K) to investigate the effect of melamine/cyanurate on cultured cells. When melamine alone or 99:1 was present, there was a 1000-fold down-regulation of the T-type voltage gated calcium channel. This may be a protective mechanism for the cells. At all concentrations, zinc finger proteins were universally up-regulated, as they have diverse functions including DNA recognition, RNA packaging, and regulation of apoptosis. When melamine alone was present at 50 mM, there was up-regulation of chemokine-like factors (IL2, IL5, IL6, IL8, and IL16).

Discussion

This study addressed some fundamental aspects of the effects of melamine on the renal system: (1) the interactions of melamine and cyanurate in urinary environments, and (2) the potential damage, cellular response, and gene activation at the cellular level when melamine/cyanurate is present.

Crystallisation of melamine (and cyanurate) is concentration dependent; a minimum 5 mM is required. This explains why foetuses and younger children are more vulnerable to melamine-tainted milk products, owing to their relatively higher intake per unit of body mass.[4]

Besides forming its own crystals, melamine can promote the formation of CaOx crystals, even at low concentrations. Therefore, even in subjects consuming small amounts of melamine-tainted milk products, stone formation (such as CaOx stone) may be observed. Accordingly, after melamine exposure it may be worth screening older children and even adults for renal stones.

The cell culture studies provided information about the effect of melamine at the cellular level. The main effect of melamine was damage to cells with the concomitant release of cell injury markers, LDH, and oxidative stress, as reported in other study.[5] This effect was also noted at the more clinically relevant 99:1 ratio of melamine and cyanurate. Physical cellular damage occurred only when melamine was present. This may explain why clinical symptoms improved after cessation of melamine intake and conservative management. However, exposure to melamine could still induce some up/down regulation of genes related to inflammatory responses. It is not known whether patients with more chronic melamine exposure had a prolonged inflammatory reaction, leading to long-term tubulo-interstitial nephritis or even renal fibrosis. To determine resolution of the problem, monitoring of some urine inflammatory markers may be relevant in patients with prolonged high-level exposure.

Based on these findings, a treatment plan can be proposed for patients with acute melamine exposure. Apart from cessation of melamine intake, adequate hydration helps decrease the urinary concentration of melamine (cyanurate) and hence formation of melamine and other urinary crystals (such as CaOx). Alkalisation of urine, by citrate and bicarbonate, may also help to inhibit melamine crystallisation. Treating active urinary tract infection may also help decrease crystal formation. The use of Chinese herbal medicines (*Shi Wei* and *Semen Coicis*) could also help reduce melamine crystallisation.

Acknowledgement

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-BS-07).

References

1. Wong SN, Chiu MC. The scare of melamine tainted milk products. Hong Kong J Paediatr 2008;13:230-4.
2. Lau HY, Wong CS, Ma JK, Kan E, Siu KL. US findings of melamine-related renal disorders in Hong Kong children. Pediatr Radiol 2009;39:1188-93.
3. Yuen JW, Gohel MD, Poon NW, Shum DK, Tam PC, Au DW. The initial and subsequent inflammatory events during calcium oxalate lithiasis. Clin Chim Acta 2010;411:1018-26.
4. Wang Z, Luo H, Tu W, et al. Melamine-tainted milk product-associated urinary stones in children. Pediatr Int 2011;53:489-96.
5. Wang C, Qin X, Huang B, He F, Zeng C. Hemolysis of human erythrocytes induced by melamine-cyanurate complex. Biochem Biophys Res Commun 2010;402:773-7.

YL Lau 劉宇隆
W Tu 涂文偉

Case-control study of Sichuan and Hong Kong children with melamine-associated renal stones: renal ultrasonography and urinary IL-8 and MCP-1 levels

Key Messages

1. The clinical features of Sichuan and Hong Kong children with renal stones differ significantly, suggesting different aetiological pathways.
2. Urinary interleukin-8/creatinine ratio was higher in children having renal stones in Sichuan, but in Hong Kong it was similar in children with or without renal stones. This suggests different inflammatory potentials.
3. A large percentage of children still had renal stones at the 1-year follow-up.

背景：2008年，中國兒童食用受三聚氰胺污染奶粉後有腎結石症狀。檢查的22 384 000名兒童中有294 000名被診斷患有三聚氰胺相關腎結石，其中51 900名全國公開報導。我們曾調查過189名患腎結石的四川患兒，發現此種情況在嬰兒更常見，來自貧困家庭的患兒病情更嚴重。這些患兒的遠期情況尚不清楚。另外，同時在香港，轉介的27 000名兒童經過腎臟超聲檢查後，有15名懷疑患有結石並上報衛生防護中心。這些腎結石的性質仍不清楚。

目的：研究假設四川患兒三聚氰胺暴露量大，患有真正三聚氰胺相關腎結石；但是香港兒童三聚氰胺暴露量小，臨床特徵可能不同，所患腎結石可能與三聚氰胺無關。在隨訪的一年中，我們比較這兩組兒童的腎臟超聲結果及尿液中炎性細胞因子的水平。

研究設計和方法：在前次研究中，四川華西第二教學醫院確診了51名三聚氰胺腎結石患兒。本次研究中納入44位作為三聚氰胺腎結石真正病例。香港的六間醫院利用腎臟超聲篩查了16 567名12歲以下的兒童，有34名被懷疑患有三聚氰胺腎結石，我們納入其中22名作為本研究的對照組。在隨訪的第6、9以及12個月，對腎臟超聲和尿液中IL-8和MCP-1水平進行了比較。

結果：香港兒童的年齡（75.0±42.1月）明顯比四川兒童（25.7±23.8月）大。四川兒童腎結石數量（1-8，中位數為４）顯著高於香港兒童（1-2，中位數為１）。四川兒童最大結石的尺寸（6.3±4.2毫米）顯著大於香港兒童（3.8±1.6毫米）。隨訪一年後，28%的四川兒童和48%的香港兒童仍有腎結石。在第６個月，仍有腎結石的四川患兒的尿液IL-8水平顯著高於結石完全排出的四川患兒，以及高於已排出或未排出結石的香港兒童（P<0.0001）。香港有或沒有腎結石的兒童之間尿液IL-8水平相似。尿液IL-8水平與最大結石的尺寸在四川兒童中具有顯著相關性（r=0.82, P<0.0001），但在香港兒童中無相關性（r=0.21, P=0.36）。在各組兒童之間，尿液MCP-1水平無明顯差異。

結論：四川和香港腎結石兒童的臨床特徵顯著不同，顯示其病因來源不同。尿液IL-8水平在四川腎結石患兒中較高，但在香港有或沒有腎結石的兒童中相似，顯示其具有不同的致炎性。

意義：在隨訪的一年中，有相當部分兒童仍患有腎結石。香港兒童的腎結石與四川患兒的腎結石在本質上不同。有必要進行長期隨訪。

Introduction

In September 2008, there was an outbreak of melamine-associated renal stones in children in China.[1] Melamine was added to infant formula to elevate protein content. According to the Chinese Ministry of Health, in December 2008, 22 384 000 children with suspected melamine exposure were examined. Of whom, 294 000 were diagnosed as having urinary stones and 51 900 were hospitalised. In our previous study, 189 (2.6%) of 7328 children who presented to the West China Second University Hospital in Sichuan with melamine exposure were identified by ultrasonography as having urinary stones.[2] Melamine-associated urinary stones occurred more frequently in infants, and children from poorer families were more seriously affected.

In response, the Hong Kong SAR Government formed an ad hoc expert group to offer advices on the clinical care of suspected cases, the public health response, research, and food safety. According to the report submitted on 15 April 2009, 56 847 children were screened in designated clinics. Of these, 27 616 were

Hong Kong Med J 2013;19(Suppl 8):S26-30

Department of Paediatrics & Adolescent Medicine, The University of Hong Kong
YL Lau, W Tu

Principal applicant and corresponding author:
Prof Lau Yu Lung
Department of Paediatrics and Adolescent Medicine, Queen Mary Hospital, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China
Tel: (852) 2255 4481
Fax: (852) 2855 1523
Email: lauylung@hku.hk

Table 1. Kidney stone size and number at presentation in Sichuan and Hong Kong patients

Parameter	Sichuan patients (n=44)	Hong Kong patients (n=22)	P value (test)
Mean±SD age (months)	25.7±23.8	75.0±42.1	<0.0001 (Unpaired t-test)
Sex			0.542 (Chi-square test)
No. (%) of males	23 (52.3)	9 (40.9)	
No. (%) of females	21 (47.7)	13 (59.1)	
Mean±SD aggregate stone index (mm)	18.2±12.1	4.3±1.7	<0.0001 (Unpaired t-test)
Mean±SD largest stone size (mm)	6.3±4.2	3.8±1.6	0.0087 (Unpaired t-test)
Median (range) stone number	4 (1-8)	1 (1-2)	<0.0001 (Wilcoxon rank sum test)

Table 2. Melamine concentration in milk consumed by Sichuan and Hong Kong patients

Brand	Patients*	Melamine concentration† (mg/kg)
	Sichuan (n=44)	
三鹿 (Sanlu)	31	>5500
南山 (Nanshan)	3	>5500
雅士利 (Yashili)	3	53.4
圣元 (Synutra)	2	150
施恩 (Scient)	4	17.0
伊利 (Yili)	1	8
雀巢 (Nestle)	1	1.4
多美滋 (Dumex)	1	0
Others	3	-
	Hong Kong (n=22)	
蒙牛(Mengniu)	16	0
伊利 (Yili)	11	5.5
雀巢 (Nestle)	3	1.4
維記 (Kowloon Dairy)	1	0
美贊臣 (Mead Johnson)	1	0
光明牌 (Bright Brand)	1	8.6

* Five Sichuan and 11 Hong Kong patients consumed more than one type of milk

† Information from General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, or Centre for Food Safety, The Government of the Hong Kong SAR. The melamine concentration of the milk consumed by the Sichuan and Hong Kong children was significantly different (P<0.0001, unpaired *t*-test).

referred to Special Assessment Centres where further tests including renal ultrasonography were performed.

Our hypothesis was that Sichuan children with heavy melamine exposure suffered from genuine melamine-associated renal stones, whereas Hong Kong children with minimal melamine exposure had different clinical features, so that their renal stones may not be melamine-related. This study aimed to compare the clinical features and the renal ultrasound findings, as well as the urinary inflammatory cytokine/creatinine ratios in the Sichuan and Hong Kong children at the 1-year follow-up.

Methods

This study was conducted from April 2009 to September 2010. In our previous study, 44 out of 51 Sichuan children admitted to the West China Second University Hospital with suspected melamine-associated renal stones were identified as genuine cases.[2] In Hong Kong, 16 567 children under 12 years of age underwent renal ultrasonography in Kwong Wah Hospital, Pamela Youde Nethersole Eastern Hospital, Queen Elizabeth Hospital, Queen Mary Hospital, Tuen Mun Hospital, and United Christian Hospital. Of them, 22 of 34 children suspected to have melamine-associated renal stones were recruited as controls. All these cases and controls were followed up using renal ultrasonography and urinary IL-8 and monocyte chemotactic protein-1 (MCP-1) at about 6, 9, and 12 months.

Results

The Sichuan children were significantly younger than the Hong Kong children (P<0.0001, Table 1), but the gender distribution was similar. The largest stone size and the number of stones at presentation were significantly greater in the Sichuan than Hong Kong children (P=0.0087 and P<0.0001, respectively). The melamine concentration in the milk consumed by Sichuan children was significantly higher (P<0.0001, Table 2).

Figure 1 shows the Kaplan-Meier plot of the presence of renal stones in Sichuan and Hong Kong children. At the 12-month follow-up, about 28% of Sichuan children and 48% of Hong Kong children still had evidence of renal stones (P=0.1302). In two Sichuan children, their renal stones were discharged, but they still had hydronephrosis at the 9- and 12-month follow-up. One Hong Kong child who had a linear echogenic focus with double-line configuration (suggestive of vascular calcification rather than renal stone) was classified as having a stone.

Figure 2 shows the urinary IL-8/creatinine and MCP-1/creatinine ratios in Sichuan and Hong Kong children at the 6, 9, 12 months follow-up. At months 6 and 9, Sichuan children with renal stones had significantly higher urinary IL-8/creatinine ratio. This suggested that melamine stones could induce renal interstitial inflammation. The urinary IL-8/creatinine ratio in Sichuan children with renal stones declined from month 6 to month 12, reaching levels similar to those whose renal stones was discharged completely. This indicated that such inflammation could largely subside, despite of the persistence of melamine stones. In Hong Kong children, the urinary IL-8/creatinine ratio was similar to that in subjects with or without renal stones. Moreover, the ratios in these two groups of Hong Kong

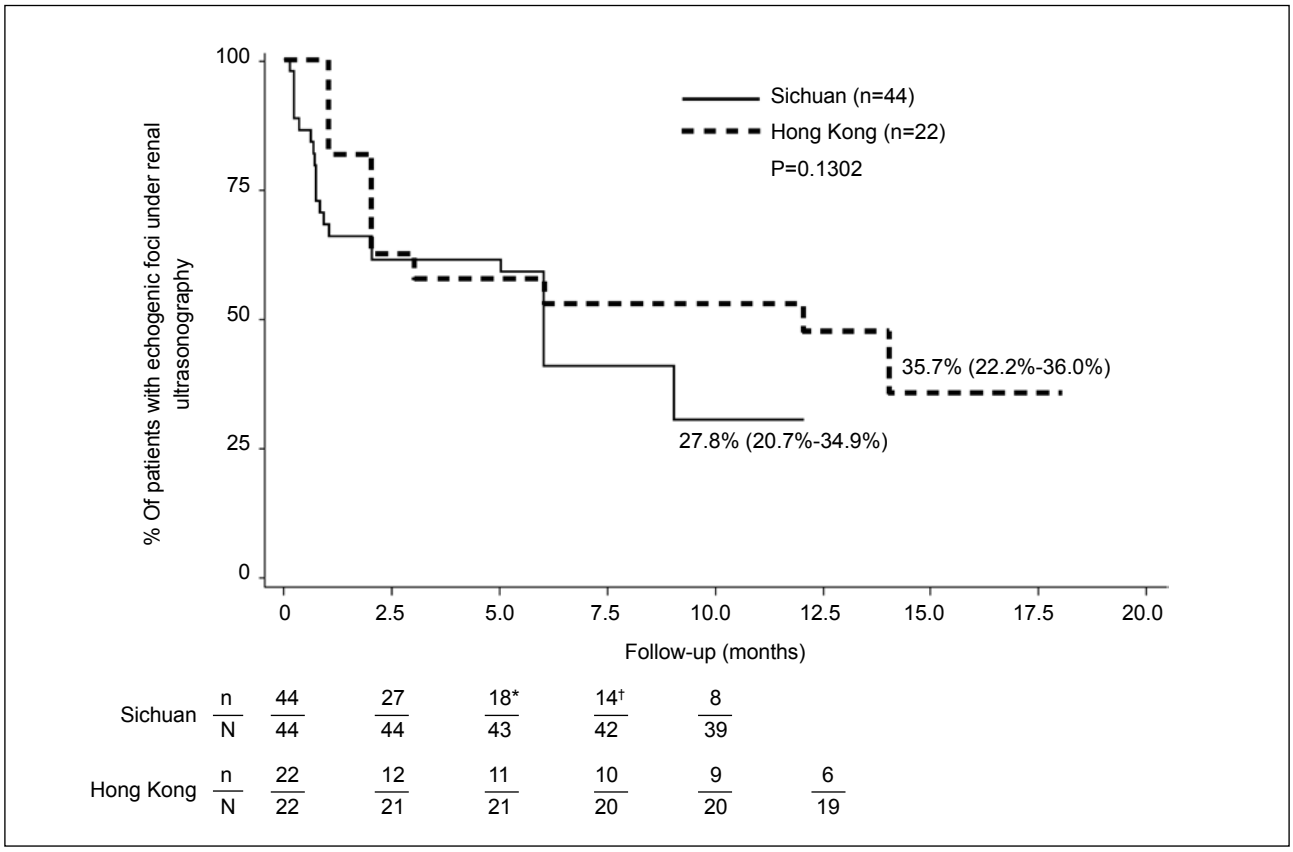

Fig 1. The Kaplan-Meier plot of the presence of renal stones in Sichuan and Hong Kong patients

* Including 14 cases with stones confirmed at 6-month follow-up and four cases with stones confirmed at previous follow-up and did not come at 6-month follow-up, but came again at subsequent follow-up

† Including 12 cases with stones confirmed at 9-month follow-up and two cases with stone confirmed at previous follow-up and did not come at 9-month follow-up, but came again at subsequent follow-up

children were similar to those of Sichuan children whose stones were discharged completely and were lower than in Sichuan children with persisting renal stones (Fig 2).

The urinary MCP-1/creatinine ratios only showed marginal significance at the 6-month follow-up in the ANOVA analysis, and not at all in the post hoc test (Fig 2).

Discussion

In Hong Kong children, the prevalence of renal stones or echogenic foci identified by ultrasonography has been reported be 0.03% to 0.6%.[3] In the present study it was 0.205%. These figures are much lower than those in Chongqing (2.51%),[4] Hangzhou (3.61%), Sichuan (2.58%), and Beijing (2.9%). In fact, among children not exposed to melamine-tainted milk products (MTMP) in Chongqing, the prevalence was about 0.41%.[4] This suggests that Hong Kong children with 'melamine-associated' renal stones had largely incidental findings not related to melamine toxicity. Even if some of these stones were related to melamine, the levels of exposure were relatively low, because Hong Kong patients were much older than Sichuan patients, and hence consumed much lower quantities of MTMP per kg body weight. In addition, the highest melamine concentration in mainland Chinese MTMP was >5500 mg/kg,[3] whereas it was much lower in Hong Kong.

The number of renal stones and the largest renal stones were significantly greater in Sichuan children than in Hong Kong children. This was presumably due to differences in the levels of melamine exposure.

The resolution rates of the renal stones at the 1-year follow-up were low in both the Sichuan and Hong Kong children (72% and 52%, respectively), compared to Beijing children (95.5%).[5] The Sichuan patients probably had more severe renal disease because of the greater number and size of stones.[2] Larger stones could not be passed out as readily as smaller stones.[2] Beijing patients[5] had less severe renal disease than the Sichuan cohort.[2] Hong Kong children had even lower resolution rate for renal stones, probably because the stones were not truly related to melamine, even they were smaller.[2]

The urinary IL-8/creatinine ratio is a useful marker for kidney inflammation secondary to melamine-associated renal stones. The absence of elevated ratios in Hong

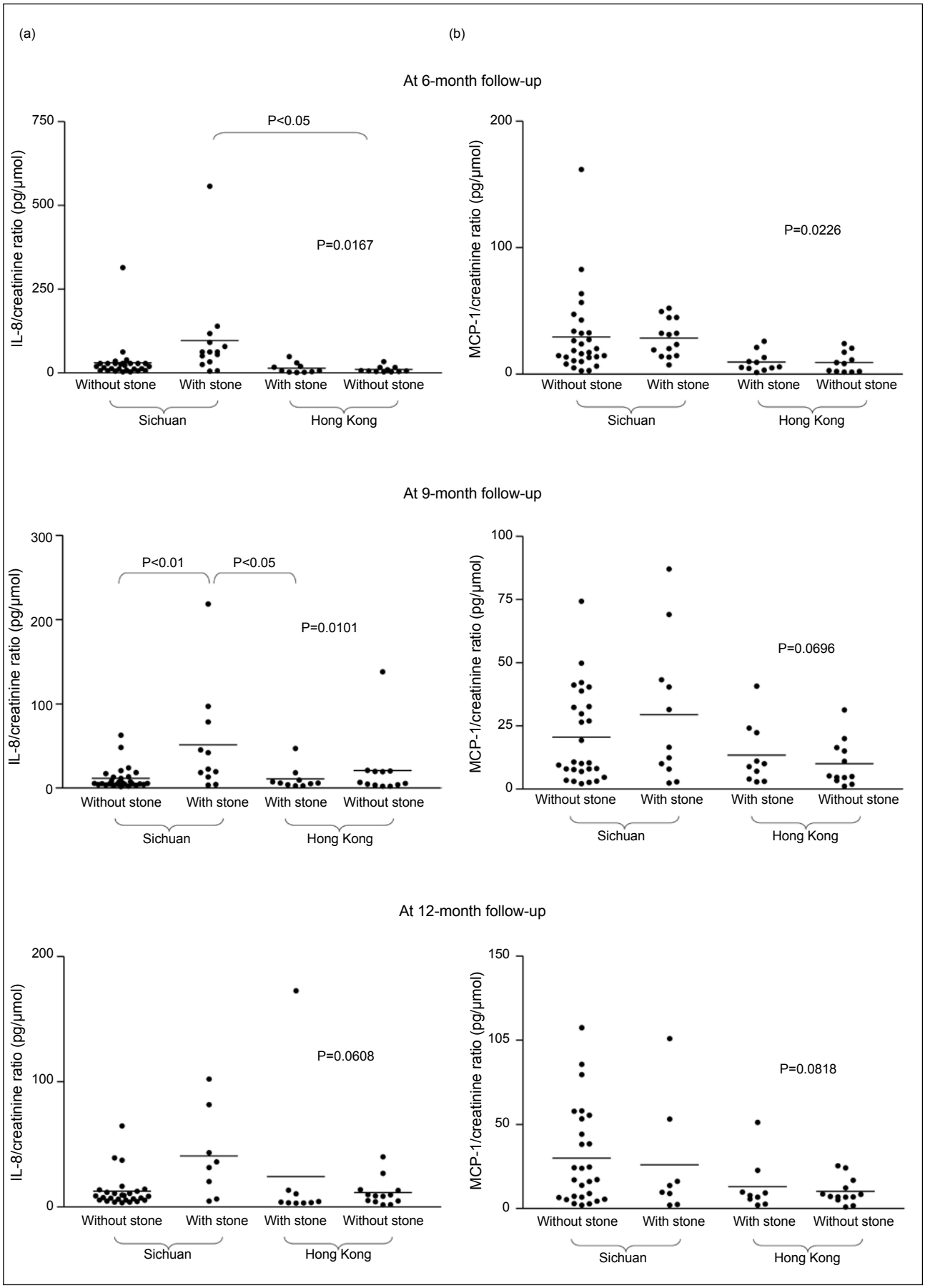

Fig 2. (a) Urinary IL-8/creatinine and (b) MCP-1/creatinine ratios in Sichuan and Hong Kong patients with or without renal stones over 1-year follow-up

Kong children with renal stones suggests that the stones may not have been truly related to melamine or that the stones were too small to cause IL-8 induction. The ratio decreased in most Sichuan children once the stones were completely discharged. This suggests that the ratio can be a means of monitoring renal interstitial inflammation. Even with persisting renal stones, the ratio in Sichuan children decreased over 1 year, suggesting that inflammation may resolve partially even if the stones persist. The ratio did not seem to be useful for monitoring renal inflammation.

Limitations of the present study were that the Sichuan cohort was recruited from inpatients, hence not representative of the general population with melamine-associated renal stones. This compromised the validity of direct comparison of clinically ill Sichuan children with Hong Kong children who were largely asymptomatic. The melamine-associated renal stones were more and larger in Sichuan outpatient children than in Hong Kong children.[2] This suggests that the renal stones in Hong Kong children may not be melamine related. In addition, the melamine concentration of the milk consumed by Hong Kong children was much lower than that consumed by Sichuan children. Some Sichuan children lived far from Chengdu, and 44 of them did not complete the 1-year follow-up. The use of the Kaplan-Meier analysis resolved this issue in some extent.

Conclusion

At the 1-year follow-up, about 28% of Sichuan children with melamine-associated renal stones still had renal stones and some evidence of renal interstitial inflammation. This figure is likely to be an overestimate for the general population of such children, as our Sichuan cohort was recruited from a more severely affected inpatient population. There was little evidence of renal interstitial inflammation in Hong Kong children with and without renal stones.

Mainland Chinese children with persistent melamine-associated renal stones should have long-term follow-up to monitor possible renal interstitial inflammation and fibrosis. For Hong Kong children with suspected 'melamine-associated' renal stones, the stones were unlikely to be related to melamine and no significant clinical harm was likely to ensue.

Acknowledgement

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-FU-08).

Results of this study were published in: Tu W, Yang H, Luo H, et al. One-year follow up of melamine-associated renal stones in Sichuan and Hong Kong. Hong Kong J Paediatr 2012;17:12-23.

References

1. Liu JM, Ren A, Yang L, et al. Urinary tract abnormalities in Chinese rural children who consumed melamine-contaminated dairy products: a population-based screening and follow-up study. CMAJ 2010;182:439-43.
2. Wang Z, Luo H, Tu W, et al. Melamine-tainted milk product-associated urinary stones in children. Pediatr Int 2011;53:489-96.
3. Lau HY, Wong CS, Ma JK, Kan E, Siu KL. US findings of melamine-related renal disorders in Hong Kong children. Pediatr Radiol 2009;39:1188-93.
4. Yang H, Wang Q, Luo J, et al. Ultrasound of urinary system and urinary screening in 14 256 asymptomatic children in China. Nephrology (Carlton) 2010;15:362-7.
5. Shen Y, Sun Q, Gao J, et al. One year follow up of the outcomes of child patients with melamine-related kidney stones in Beijing and surrounding provinces in China. Nephrology (Carlton) 2011;16:433-9.

WT Wong 黃永德
XY Tian 田小雨
CW Lau 劉志偉
YX Wang 王毅翔
J Liu 劉　健
WS Cheang 鄭慧珊
ZY Chen 陳振宇
CS Mok 莫全城
CM Lau 劉秋銘
Y Huang 黃　聿

Renal and vascular function in pregnant and neonatal rats exposed to melamine and related compounds

許多嬰兒因長期服食含有三聚氰胺的奶製品導致腎臟中毒和腎結石。這些引起患兒腎功能出現病理變化的機制仍然不清楚。為了了解含三聚氰胺及其代謝產物的奶類製品對於健康的影響，我們試圖通過動物研究來解答以下幾個相互關聯的問題：（1）長期每日攝取低劑量三聚氰胺或其代謝產物三聚氰酸會否損傷血管及腎功能。如果可以，三聚氰酸有否在三聚氰胺所造成的損傷中起一定的作用？（2）如果單獨攝取三聚氰胺或三聚氰酸不能損傷腎功能，那麼同時攝取二者會否導致腎臟損傷？（3）孕婦長期攝取三聚氰胺和／或三聚氰酸會否導致出生後胎兒腎功能障礙？因此本研究旨在闡明：（1）嬰兒時期長期低劑量攝取三聚氰胺或三聚氰酸導致成年後的血管功能及腎功能障礙；（2）通過應用血管功能學方法檢測離體腎動脈活性，應用核磁共振檢測腎臟血流量，分析炎性因子及纖維化因子的表達，免疫組織化學以及測量血漿和尿液中三聚氰胺的含量這些方法綜合研究孕鼠長期攝取三聚氰胺或三聚氰酸導致胎兒出生後出現腎功能障礙。本研究提出了三聚氰胺損傷腎臟的新機制：三聚氰胺顯著降低腎臟血流量，損傷腎臟血管功能並且增加腎血管和腎臟炎症因子，轉化生長因子β1，骨形態發生蛋白4和環氧合酶2的含量。三聚氰胺同時能導致腎臟纖維化和炎症。本研究也為三聚氰胺及其代謝產物如何導致乳鼠腎功能障礙以及胎鼠長期攝取三聚氰胺及其代謝產物對其出生後腎臟功能有何影響提供了部分科學依據，支持了最近觀察到的嬰兒長期攝取含三聚氰胺及其代謝產物的奶製品會導致腎結石及急性腎衰竭這一臨床症狀。

Key Messages

1. In rats, long-term exposure to melamine impairs renal blood flow and renal vascular function in a dose-dependent manner.
2. Melamine-induced renal vascular dysfunction is caused by enhanced vasoconstriction and reduced vasodilatation owing to the endothelial cell dysfunction of renal arteries.
3. Endothelial dysfunction after melamine treatment is mediated through oxidative stress-dependent activation and up-regulation of cyclooxygenase 2, which produces prostanoids that act on the thromboxane receptor.
4. Transforming growth factor β1 and bone morphogenic protein 4 contribute to renal fibrosis induced by oral administration of melamine.
5. Exposure to melamine during pregnancy and lactation exaggerates renal vascular dysfunction in the offspring.

Hong Kong Med J 2013;19(Suppl 8):S31-3

Institute of Vascular Medicine, Li Ka Sing Institute of Health Sciences, School of Biomedical Sciences, The Chinese University of Hong Kong
WT Wong, XY Tian, CW Lau, J Liu, WS Cheang, Y Huang
Department of Diagnostic Radiology and Organ Imaging, The Chinese University of Hong Kong
YX Wang
School of Life Sciences, The Chinese University of Hong Kong
ZY Chen
Government Laboratory, Hong Kong
CS Mok, CM Lau

Principal applicant and corresponding author:
Prof Yu Huang
School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong SAR, China
Tel: (852) 2609 6787
Fax: (852) 2603 5022
Email: yu-huang@cuhk.edu.hk

On 11 September 2008, news media reported that thousands of infants and children in Mainland China had suffered from kidney stones and kidney failure and were hospitalised after consuming milk products contaminated with melamine. Excessive exposure to melamine caused the formation of kidney stones and thus severe obstruction of the urinary tract. Four infants lost their lives because of acute renal failure associated with melamine intake. This news caused a widespread public outcry and health scare worldwide. Melamine (1,3,5-triazine-2,4,6-triamine) derived from urea is rich of nitrogen, which was deliberately added to milk products to boost their protein contents on standardised tests that use nitrogen content as a surrogate for protein.

There were few reports on the toxicity of melamine in humans before the outbreak of the melamine incident. Limited research showed that in rats, the toxic dose causing 50% of exposed animals to die was 3.1 g/kg of melamine and 7.7 g/kg of cyanuric acid. The United States Food and Drug Administration has adopted tighter recommendations on the tolerable daily intake (TDI) for melamine, which reduced from 0.63 mg/kg to the present 0.063 mg/kg, as infants may be more susceptible than adults. In addition, both the European Food Safety Authority and the World Health Organization set the TDI for melamine at 0.2 mg/kg body weight/day. The government of the Hong Kong Special Administrative Region has also promptly set a legal limit for melamine in food. The amended Harmful Substance in Food Regulation has limited the level of melamine and related analogues to <1 mg/kg milk and food intended for consumption principally for children under the age of 36 months and by pregnant or lactating women.

There are limited studies examining the effects of melamine and its analogue cyanuric acid in experimental animals (mice, rats, cats, and dogs). Dietary exposure to melamine was found to induce calculi formation, inflammatory responses, and hyperplasia in the urinary bladder. In dogs, melamine crystalluria was also observed. Renal toxicity of melamine was reported in a study involving

chronic feeding of melamine to female rats, in which there was dose-related accumulation of calculi in the proximal tubules and chronic inflammation of the kidney. Several subchronic oral toxicity studies revealed that cyanuric acid caused renal injury (such as necrosis or hyperplasia of the tubular epithelium, neutrophilic infiltration, mineralisation, and fibrosis), which are associated with the formation of cyanurate crystals. Furthermore, the outbreak of acute kidney failure in cats and dogs in 2007 was related to co-ingestion of both melamine and cyanuric acid.[1] Both compounds may form a complex of very low solubility called melamine cyanurate, which leads to the formation of kidney stone and eventually causes tubular blockage and renal damage.[2]

The outbreak and consequence of nephrotoxicity and kidney stone in infants previously exposed to melamine-contaminated milk products had caused widespread health panic, particularly in parents and pregnant women. Nevertheless, little was actually known about the mechanism leading to pathophysiological alterations in renal function in affected children. To respond to public concerns about the safety of melamine and its related compounds in dairy products, we examined whether (1) neonatal ingestion of melamine causes impaired renal and vascular function in adult rats, and (2) ingestion of melamine during pregnancy affects the renal function of their offspring.

In adult male Sprague Dawley rats, oral ingestion of melamine in drinking water at three dosages (60, 300, and 600 mg/kg body weight/day) for 3 months did not affect body weight or systolic blood pressure, as measured by the tail-cuff method. Melamine consumption at medium and high dose markedly increased its levels in plasma and kidney tissues. Renal cortical blood flow (as measured by magnetic resonance imaging using a 3T clinical whole-body imaging system) diminished after melamine exposure. Melamine ingestion progressively impaired endothelium-dependent relaxations in the isolated rat renal arteries revealed by microvessel myography. In the presence of each of the following drugs: cyclooxygenase-2 inhibitor NS398, the thromboxane prostanoid receptor antagonist S18886, and the reactive oxygen species scavenger tiron plus DETCA, even after medium and high doses of melamine ingestion, such endothelium-dependent relaxation was significantly improved. Melamine ingestion also increased (in a dose-dependent manner) fibronectin accumulation in glomeruli, which was revealed by immunohistochemcal staining and indicated unfavourable remodelling of the kidneys and renal arteries. Increased expression of fibronectin was confirmed by Western blotting results in both kidneys and renal arteries. In addition, the expression of several pro-inflammatory biomarker proteins (such as transforming growth factor-β1, bone morphogenic protein-4, and cyclooxygenase-2) also appeared up-regulated in the kidneys and renal arteries of melamine-treated rats.

In 2-month-old female rats treated with melamine for 2 weeks before mating, their offspring were then given melamine or its vehicle for another 3 months. The male offspring born from high-melamine-dose-exposed mothers attenuated endothelium-dependent relaxation and exaggerated endothelium-dependent contractions in the presence of nitric oxide synthase inhibitor in the renal arteries, as compared to those received the vehicle control.

Findings of the present study on rats regarding the renal and vascular toxicity of melamine ingestion include (1) short-term exposure to melamine and cyanuric acid alone did not cause mortality; (2) short-term exposure to melamine and cyanuric acid combined led to significant reduction of renal blood flow, visible crystal formation in the renal cortex and medulla, and deaths; (3) long-term ingestion of melamine (600 mg/kg/day) impaired function in intralobar renal arteries with an internal diameter of about 200 μm, through activation of thromboxane prostanoid receptors, indicating possible inflammatory events leading to the production of cyclooxygenase-derived prostaglandins in the renal blood vessels; (4) renal fibrosis developed in melamine-treated rats; and (5) melamine and cyanuric acid accumulated in circulating blood and the kidneys, as revealed by mass spectrometry. These results provide useful information on the pathophysiology of events related to melamine exposure and toxicity.

The present study also showed that the oral administration of high doses of melamine or cyanuric acid alone did not induce acute toxicity in adult rats even though there was crystal formation in the renal medulla. By contrast, long-term exposure to melamine for 3 months did reduce renal blood flow and impaired renal vascular function. Although the health impact of melamine on adults is not fully understood, the concentration of urinary melamine is positively correlated with the risk of kidney stones.[3] In addition, the combination of melamine and cyanuric acid resulted in severe stone formation and renal toxicity, causing death within a few days, because melamine stones mainly affect renal tubules. In rats, the toxic effects of combined exposure to melamine and cyanuric acid are more severe.

Vascular function of the renal arteries affects renal function through the regulation of renal blood perfusion as well as glomerular filtration. Histologically, the kidney disease, the presence of melamine stones, and an increase of serum creatinine level (indicating impaired renal function) are indicative of the renal toxicity of melamine.[1] Melamine ingestion progressively impairs endothelial cell function, and augments endothelium-dependent contractions in intralobar renal arteries. These harmful effects may well contribute to the reduction of renal blood perfusion. The possible underlying mechanisms may involve increased expression and activity of pro-inflammatory signalling molecules (reflected by the increased expression of transforming growth factor-β1, bone morphogenic protein-4, cyclooxygenase-2, and fibronectin). These

modulators could become therapeutic targets in the fight against melamine-induced nephropathy.

In the present study, exposure of rat mothers to melamine exaggerated melamine-induced renovascular dysfunction in their offspring suggested that melamine was transferred maternally through gestation or lactation. Previous studies also validated the possibility of transfer through the placenta and milk.[4] Taken together, existing evidence definitely raises health concerns about melamine-contaminated food for mothers.

The present study also provided useful experimental results regarding the harmful effects of ingesting melamine, cyanuric acid, or both on the renal function of neonatal/postnatal rats. We showed that melamine and its related compounds dose-dependently impaired renal and vascular function. This was accompanied with the development of interstitial fibrosis and eventual renal damage, through activation of inflammatory pathways or overproduction of reactive oxygen species, followed by decreased bioavailability of nitric oxide. The present study also provided better understanding of the safety profile of long-term exposure of neonatal or pregnant rats to low levels of melamine and related compounds on renal and vascular function in adulthood or in their offspring.

Therapeutic interventional experiments are warranted to provide scientific support for the use of relevant drugs to alleviate the renal toxicity associated with melamine ingestion. Although no effective means are available to dissolve these kidney stones, oral potassium citrate or sodium bicarbonate are clinically used to alkalinise the urine to dissolve uric acid stones. Rats with tubular crystallisation can be treated with either potassium citrate or sodium bicarbonate, with monitoring of the size and location of crystals and renal function. Moreover, the calcium level in urine can be assessed. If ingestion of melamine and related compounds causes hypercalciuria, diuretics such as thiazides may have the potential to lower urine calcium levels.

Acknowledgement

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-BS-12). Some of the results were presented in a conference in December 2010 and at the Health Research Symposium 2010: Improving Health and Recognising Excellence on 11 September 2010.

References

1. Puschner B, Poppenga RH, Lowenstine LJ, Filigenzi MS, Pesavento PA. Assessment of melamine and cyanuric acid toxicity in cats. J Vet Diagn Invest 2007;19:616-24.
2. Lam CW, Lan L, Che X, et al. Diagnosis and spectrum of melamine-related renal disease: plausible mechanism of stone formation in humans. Clin Chim Acta 2009;402:150-5
3. Liu CC, Wu CF, Chen BH, et al. Low exposure to melamine increases the risk of urolithiasis in adults. Kidney Int 2011;80:746-52.
4. Chan JY, Lau CM, Ting TL, et al. Gestational and lactational transfer of melamine following gavage administration of a single dose to rats. Food Chem Toxicol 2011;49:1544-8.

H El-Nezami
PKH Tam 譚廣亨
Y Chan 陳　嚴
ASY Lau 劉錫賢
FCC Leung 梁志清
SF Chen 陳　峰
LCL Lan 藍傳亮
MF Wang 王明福

Impact of melamine-tainted milk on foetal kidneys and disease development later in life

背景：多種食品受三聚氰胺及其相關的物質的污染，政府有需要確保消費者的食物安全。
目標：評估胎兒接觸三聚氰胺和它的降解產物三聚氰酸的可能性，及接觸後對胎兒可能造成的後果。
方法：四個部分的目標如下：（1）使用細胞培養模型來研究三聚氰胺和三聚氰酸的交互細胞毒性；（2）檢測導致腎臟炎症和體內形成腎結石的能力；（3）利用人類胎盤灌注模型來評估母親與胎兒之間經胎盤的傳遞；和（4）透過動物模型闡明接觸三聚氰胺和三聚氰酸後，胎兒在子宮內及其早期生命階的短期與長期的涵義。
結果：三聚氰胺和三聚氰酸之間沒有出現任何加成效應或協同的交互作用。三聚氰胺能穿透胎盤到達胎兒，並在胎兒體內積聚，而三聚氰酸則沒有出現這種情況。在接受有三聚氰胺和三聚氰酸的老鼠胚胎內，並未發現三聚氰胺晶體或可觀察到的的毒性。
結論：三聚氰胺能穿過人類胎盤。
涵義：孕婦接觸三聚氰胺和三聚氰酸後，這些物質能傳遞至成長中的胎兒。由於污染物對人類具毒性，接觸後對胎兒可能會構成重大的風險，因此三聚氰胺的毒性機制在胎盤中造成的毒性影響需要進一步研究。

Key Messages

1. Melamine has very low cytotoxicity on kidney cells of a susceptible species (canine), and the presence of its main degradation product cyanuric acid did not influence its cytotoxicity. This indicates that the toxicity of melamine is mainly due to the formation of kidney stones rather than a direct toxic effect on kidney cells.
2. Ingestion of melamine alone failed to induce kidney stones even under conditions of restricted drinking water access. In mice administrated with melamine together with cyanuric acid, no renal stones were formed when the supply of drinking water was unrestricted. However, when drinking water was limited, stone formation was observed.
3. Melamine was detected in the foetal circulation of perfused human placenta and easily transferred through it. The presence of cyanuric acid did not influence the transfer of melamine from the maternal to the foetal side.
4. Administration of melamine and cyanuric acid to pregnant mice did not cause any noticeable developmental or reproductive abnormalities.
5. No melamine crystals were detected in kidneys of the embryos or breastfed pubs, despite the mothers having melamine crystals in their kidneys and renal failure.

Hong Kong Med J 2013;19(Suppl 8):S34-8

The University of Hong Kong:
School of Biological Sciences
H El-Nezami, FCC Leung, SF Chen, MF Wang
Department of Surgery
PKH Tam, Y Chan, LCL Lan
Department of Paediatrics and Adolescent Medicine
ASY Lau

Principal applicant and corresponding author:
Dr Hani El-Nezami
The University of Hong Kong, School of Biological Sciences, Kadoorie Biological Sciences Building, S5-13, Pokfulam, Hong Kong SAR
Tel: (852) 2299 0835
Fax: (852) 2299 0364
Email: elnezami@hku.hk

Introduction

Consumption of melamine-tainted milk products has affected tens of thousands of Chinese children. Up to 27 November 2008, 294 000 children were reported to show urinary system abnormalities, of whom 51 900 were hospitalised. It is uncertain whether such children will incur other complications such as tumourigenesis or growth retardation in the future.

Due to immaturity and quickly developing organs, foetuses may be highly susceptible to the effects of environmental toxins.[1-4] There is association between growth and health of the foetus and infant and the risk of several diseases later in life. Transplacental transfer of toxic compounds via the human placenta is important for foetal risk assessment. Studies on human placenta are crucial because of functional differences in placental anatomy and physiology between different species.

In China, infant milk formula contaminated with melamine and cyanuric acid (CA) was taken by the children. These compounds have been found in food products (milk and other dairy products, eggs, chicken) that were consumed in high amounts. There is potential toxicity from consuming melamine in combination with its degradation products. This study aimed to investigate the impact of possible synergistic effects between melamine and CA and the potential of transplacental passage of melamine and its consequences on foetuses and disease development later in life.

Methods

This study was conducted from April 2009 to December 2011 and was divided into four parts to study: (1) interactive cytotoxicity of melamine and CA using Madin-Darby canine kidney cells, (2) induction of renal inflammation and kidney stones in vivo in mice, (3) the maternal foetal transplacental passage using an

ex vivo human placenta perfusion model, and (4) short and long-term implications of exposure to melamine and CA in utero and during early stages of life in a mice model.

Results

Study I: interactive toxicity of melamine and its degradation product in a cell culture model

When melamine was mixed with CA in different ratios of 1:1, 10:1, 100:1, and 1000:1, and then incubated with Madin-Darby canine kidney cells for 48 hours, there was a very weak cytotoxic effect (as measured by the cell viability); less than 20% of the cells were adversely affected. The data obtained from the cytotoxicity assays of melamine and CA was compared with the effect of melamine and CA alone (Fig 1).

Study II: induction of renal inflammation and kidney stones in vivo in mice

In mice, ingestion of melamine alone failed to induce kidney

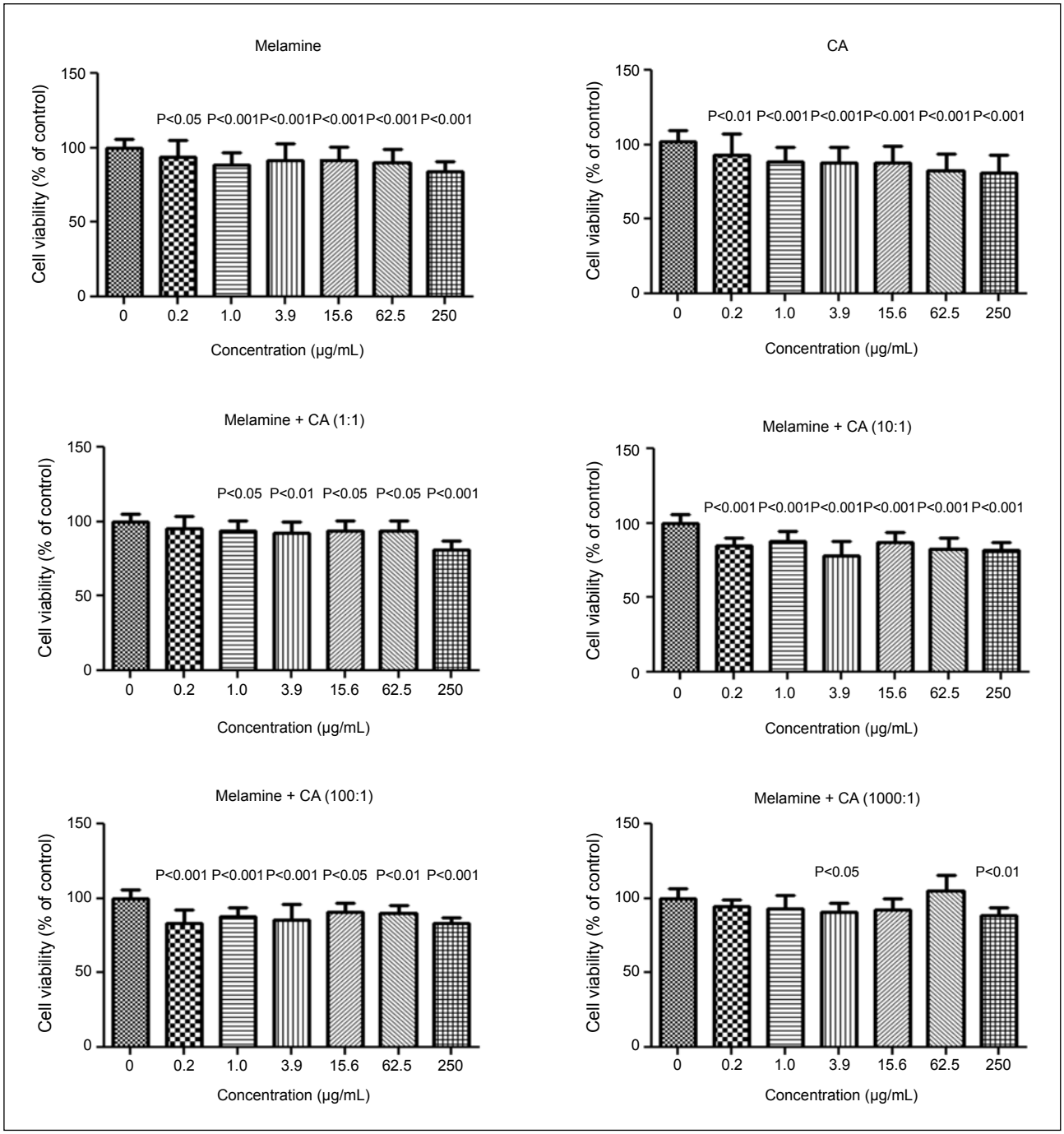

Fig 1. Individual and combined cytotoxicity of melamine and cyanuric acid (CA) after 48 hours of exposure. At least two independent experiments with six data points were run for each test chemical

stones even under conditions of restricted drinking water. When melamine was administrated with cyanuric acid for 3 days, no renal stones were formed when drinking water was unrestricted, but when drinking water was limited, stone formation accompanied by high levels of serum urea and creatinine were observed (Fig 2).

Study III: maternal foetal transplacental passage using an ex vivo human placenta model

When placentas were perfused with 10 μM melamine, approximately 0.15% of that concentration was detected in the foetal circulation after 5 minutes. The amount of melamine increased in the foetal circulation and decreased in the maternal circulation over time and at the end of the 4-hours perfusions, a mean of 62% and 39% of the original melamine concentration were detected in the maternal and foetal circulation, respectively.

As a rat study indicated that melamine transfer through placenta is dose dependent,[5] additional perfusion experiments with 1 mM melamine were carried out to investigate whether there was a difference in the transfer based on concentrations. Transfer of 1 mM melamine did not differ significantly.

Because melamine contaminated products may contain CA, perfusions were carried out with a mixture of 10 μM melamine with 10 nM of CA, to investigate whether CA affected the transfer of melamine. Transfer of melamine in these perfusions was similar, irrespective of the CA concentrations.

Study IV: short- and long-term implications of exposure to melamine

There were no significant changes in the number of mouse embryos in the control and melamine mothers. However, embryos in the latter mothers were slightly smaller in size. Morphological and histological examination of the embryos revealed no obvious bleeding or other abnormalities, and the blood vessels were well developed (Fig 3).

Discussion

Although the pathophysiology of urinary stones secondary to exposure to both melamine and CA has been reported, their combined action on kidney cells remains unknown. Study I screened for interactive toxicity of melamine and CA using a cell culture model. No additive or synergistic interactions were noted when mixed in ratios of 1:1, 10:1, 100:1, and 1000:1. Melamine generally appeared to reduce the harmful acidic effects induced by CA.

The effects of melamine and CA uptake on kidney stone formation have been reported in fish, cats, dogs, and pigs, but not in mice. Results from study II and others indicated that melamine administration alone without CA cannot induce the renal stone formation in experimental animals. When melamine and CA were administered together for 3 days, melamine crystals were found in the kidneys together with acute renal failure. However, this only occurred when access to drinking water was limited. Under conditions of unrestricted drinking water, ad libitum crystals were found very occasionally, and no dilated tubules were observed in

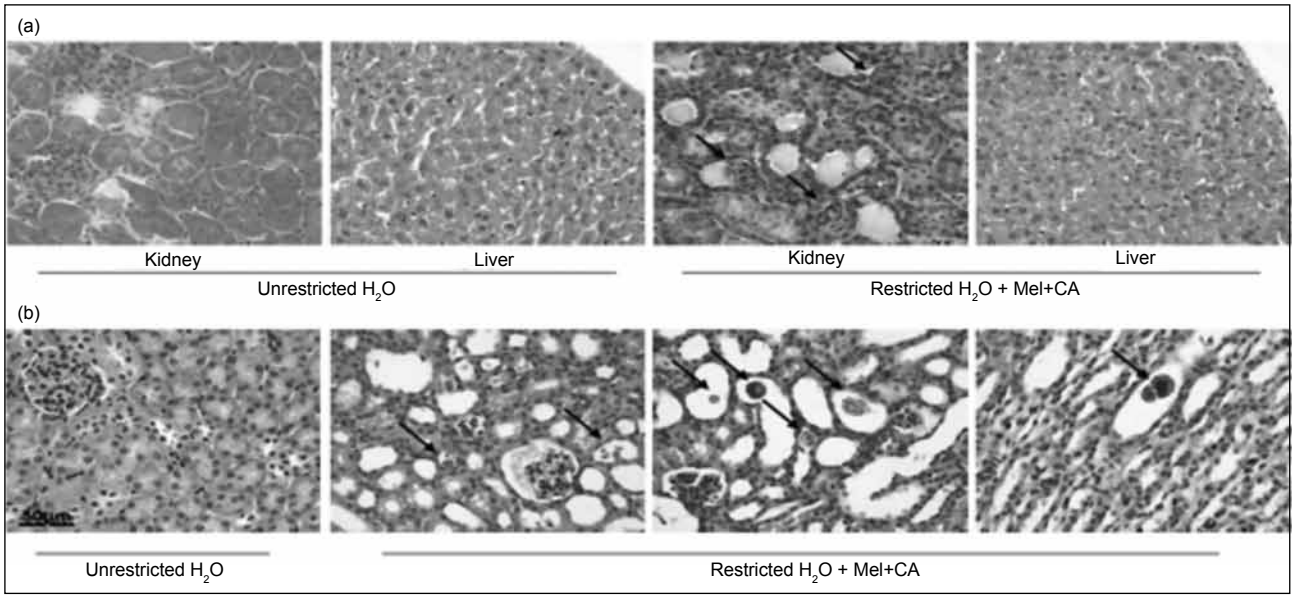

Fig 2. Histology analysis of melamine stone formation in the kidney in frozen section stained with H&E: (a) frozen sections of the kidney and liver from mice fed with melamine and cyanuric acid (CA) under restricted access to drinking water (Restricted H_2O + melamine + CA) were compared to water delivered ad libitum (Unrestricted H_2O). (b) Paraffin sections of mouse kidneys under restricted access to drinking water were compared to unrestricted drinking water controls. From left to right, cortical regions of Unrestricted H_2O group and the glomeruli, tubules and medullary regions of the Restricted H_2O + melamine + CA group. Scale bar represents 50 μm. Melamine stones are indicated with arrows

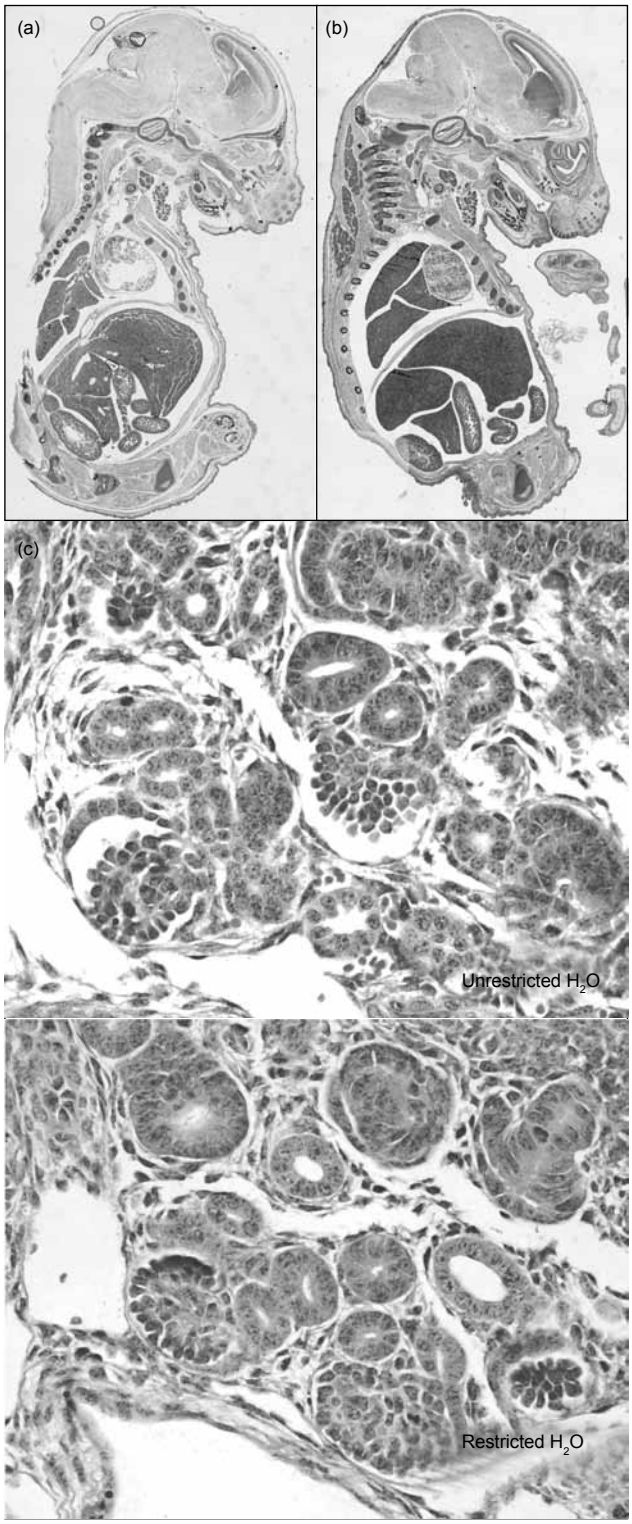

Fig 3. Cross-sections of mouse embryos stained with H&E: (a) pregnant mouse control and (b) pregnant mouse administrated with melamine and cyanuric acid (400 and 40 mg/kg/day for 3 days). (c) Cross-sections of embryo kidneys from pregnant mouse administrated with melamine and cyanuric acid

tissue sections and the mice had no symptoms of kidney failure. The half life of melamine in the blood is about 2.7 hours and it is cleared mainly through renal system. After restricting drinking water, melamine and CA were retained longer with higher concentrations in the kidney, enabling the two chemicals to interact and form crystals. In addition, the reduction of kidney function by crystals further limited the water exchange and the kidney failure occurred rapidly. Our results partially explained how melamine stones cause acute kidney failure in patients and animal studies.

During pregnancy, the placenta develops and its thickness and cell layers decrease from >50 μm at the 2nd month to <5 μm at the 37th week of pregnancy. Due to the changes in placenta, transplacental transfer probably varies during the course of pregnancy and because it is thinnest at term, drug transport may also be highest.[6] Study III is the first report on melamine transfer on human placental perfusion and provides the evidence of a fast transfer through the term human placenta. Melamine crossed the placental barrier quickly, as indicated by the presence of small concentrations (0.12 to 1.34%) in the foetal circulation 5 minutes after the addition to the maternal circulation; concentrations exceeded 34% after 4 hours of perfusion. One study on the transfer and accumulation of melamine in rat foetuses and placentas suggested that the transfer of melamine is dose dependent.[5] In our study, there was some indication of slightly quicker transfer with higher concentrations, but the difference was not significant. The kinetics of melamine clearly differed from those of antipyrine, which diffuses passively through the placenta. Transfer of melamine was significantly slower, implicating the involvement of other contributing factors such as the presence of placental efflux transporters.

Results from study IV using mouse and alternative water supply to investigate the effect of melamine and CA on embryo development suggested that stones might only be formed in a functioning kidney. As embryonic kidney has no function, hence no stone was detected in embryos from pregnant mice exposed to melamine and CA.

Acknowledgements

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-BS-16). We thank Pro Kirsi Vahakangs, Prof Seppo Auriola from the School of Medicine, University of Eastern Finland, Prof Seppo Heinonen and staff of the maternity ward in Kuopio University hospital for the placental perfusion studies.

References

1. Neri M, Ugolini D, Bonassi S, et al. Children's exposure to environmental pollutants and biomarkers of genetic damage. II. Results of a comprehensive literature search and meta-analysis. Mutat Res 2006;612:14-39.
2. Perera FP. Children are likely to suffer most from our fossil fuel addiction. Environ Health Perspect 2008;116:987-90.
3. Godschalk RW, Kleinjans JC. Characterization of the exposure-

disease continuum in neonates of mothers exposed to carcinogens during pregnancy. Basic Clin Pharmacol Toxicol 2008;102:109-17.
4. Wild CP, Kleinjans J. Children and increased susceptibility to environmental carcinogens: evidence or empathy? Cancer Epidemiol Biomarkers Prev 2003;12:1389-94.
5. Jingbin W, Ndong M, Kai H, Matsuno K, Kayama F. Placental transfer of melamine and its effects on rat dams and fetuses. Food Chem Toxicol 2010;48:1791-5.
6. Vahakangas K, Myllynen P. Experimental methods to study human transplacental exposure to genotoxic agents. Mutat Res 2006;608:129-35.

AUTHOR INDEX

Published by the Hong Kong Academy of Medicine Press for the Government of the Hong Kong Special Administrative Region. The opinions expressed in the *Hong Kong Medical Journal* and its supplements are those of the authors and do not reflect the official policies of the Hong Kong Academy of Medicine, the Hong Kong Medical Association, the institutions to which the authors are affiliated, or those of the publisher.