

CF Ng 吳志輝
KLE Hon
MDI Gohel

Effects of melamine on urine crystallisation kinetics and cell responses

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.

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。檸檬酸和碳酸氫鹽治療是有效的。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。

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 (37°C) 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 ($\mu\text{m}/\text{minute}$), 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 alkalinisation 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 fetuses 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). Alkalinisation 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.