

Lessons Learnt from the Melamine Incident of Paediatric Stones: Toxicity and Inflammatory Response of Melamine Cyanurate

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ABSTRACT

News regarding drinking milk products contaminated with melamine had gained considerable coverage. The most affected victims had been infants and children with kidney being the most affected organ. There were challenges in detecting and diagnosing renal stones in infants. Paediatric stone disease differs considerably in many aspects from that in adults, with a prevalence of 0.5 cases per 1000 (compared to 70 per 1000 in adults). We attempted to investigate the early cellular events of kidney cells in response to melamine and related lithogenic ions. A two-compartment transwell culture with human kidney cortical WT 9-12 cell line allowed us to investigate the melamine crystals interacting with the apical surface. In response to the clinically relevant ratio of melamine and cyanurate, i.e. 99:1 (v/v), 25% of the cells were destroyed to demonstrate significant disturbance to the tight junction monolayer of cortical epithelium. In this connection, cytokines were detected, in both the apical and basolateral compartments of the culture. Current results indicated that, apart from cytotoxicity, melamine cyanurate induced inflammatory response at protein and gene levels. In particular, IL-5 is regarded as a Th-2 cytokine that stimulates B cell growth and increases immunoglobulin secretion, suggesting a shift towards humoral response. Case studies and stone analysis revealed the stones to be of pure uric acid.

Key words: *Gene microarray; Humoral response; Melamine; Paediatrics; Uric acid.*

INTRODUCTION

Renal stones in young children, especially infants, are rarely seen. In the late 2008, melamine had caused the increase of renal stone incidences in infants and children in Mainland China^{1,2}. In Hong Kong, over 40,000 children had been screened for the melamine-related stones, and 15 cases were reported positive³. Most children afflicted with melamine-related stones were described as asymptomatic until renal abnormalities, revealed by impaired renal function was so severe that melamine and its crystalline stone had done its damage on first clinical presentation⁴. Whilst most infants and children affected were less than 3-years old, the long-term effects and damage are largely unknown. There are numerous evidences that the presence of crystals, such as calcium oxalate mainly from diets, may cause renal cell and tissue damage⁵ as well as elevating cytokines⁶ and mediators of inflammation⁷ including increase in gene expression. The cytotoxicity of melamine and cyanurate is currently unknown at the level of gene expression, cytokine activation and inflammatory markers. The case study presented in this paper demonstrates that children affected with suspected melamine cyanurate “poisoning” may have a more complex and heterogeneous event occurring in the renal tubules. At the same time, laboratory studies, using the human tubular cell line in an established transwell-insert

model⁸, allowed us to investigate the direct impact of the forming crystals with the cells in regards to damage, endocytosis, toxicity and/or inflammatory responses.

PATIENT RECRUITMENT

Case #1: A 7-year old male was admitted in Accident and Emergency of Prince of Wales Hospital (PWH). Known to have been drinking milk containing melamine (confirmed), stopped drinking one week before admission.

Clinical Presentation: Swelling, Oliguria, pain on urination. Low urinary output and suspected of “urinary tract infection”.

Laboratory findings: Urea (6.1 mmol/L) and creatinine (67 μ mol/L) were increased and microalbuminuria was observed. Renal ultrasound showed high echogenicity, suggesting renal stones.

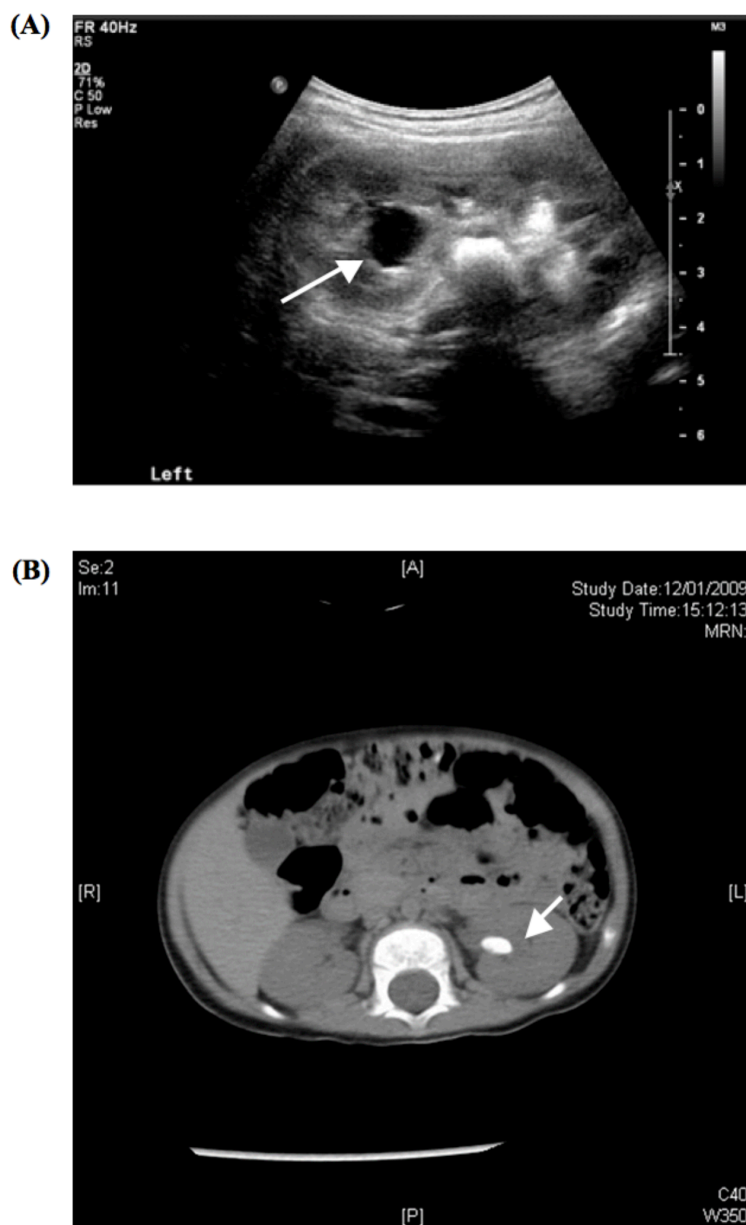
Clinical Management: Hydration and urine alkalization was started and resolved without any surgery, lithotripsy and / or further treatment after a month.

Case #2: A 1-year old boy admitted in hospital in China and brought over to Hong Kong at PWH for treatment. He had consumed milk containing melamine (confirmed) for past 6 months.

Clinical Presentation: Swelling, oliguria. Performed Percutaneous nephrostomy but not successful and had open surgery and stone removed in Mainland China hospital. On arrival at PWH, the child was having normal development and blood pressure. No prior biochemical data available or stone data. Urine test on admission at PWH

showed presence of blood and trace protein in urine. All other indices were normal. Kidney-Ureter-Bladder X-ray indicated no abnormality. Ultrasound showed “left renal pelvic stone with moderate hydronephrosis” (Fig. 1(a)). Non contrast CT-scan confirmed the diagnosis (Fig. 1(b)).

Figure 1 (A) Ultrasound and (B) non-contrast CT scan of the patient in case #2. The arrows show the “suspected” stone.



Laboratory findings: No biochemical work-up available. Ultrasound showed bilateral stones and immediate to surgery.

Clinical Management: Extracorporeal Shock Wave Lithotripsy (ESWL) was performed once and the stones did not fragment well. An attempt to cannulate via Percutaneous Nephrolithotomy (PCNL) was not successful. Finally, open pyelolithotomy

was done and the stones were completely cleared. Stone fragments (Fig. 2) were sent for analysis to Prof. Gernot Schubert at the Vivanter Klinikum, Berlin, Germany. Hydration and alkalization therapy was continued until discharged.

Findings: Stones were analysed to be 100% uric acid.

Figure 2:

Stone fragments retrieved from Patient #2 after open pyelolithotomy and sent for analysis.



MATERIALS AND METHODS

The two-compartment transwell culture system

This study was done based on our earlier findings of the interactions of calcium oxalate crystal interactions with human proximal cells in culture. The procedure is similar to the one published before⁸. Briefly,

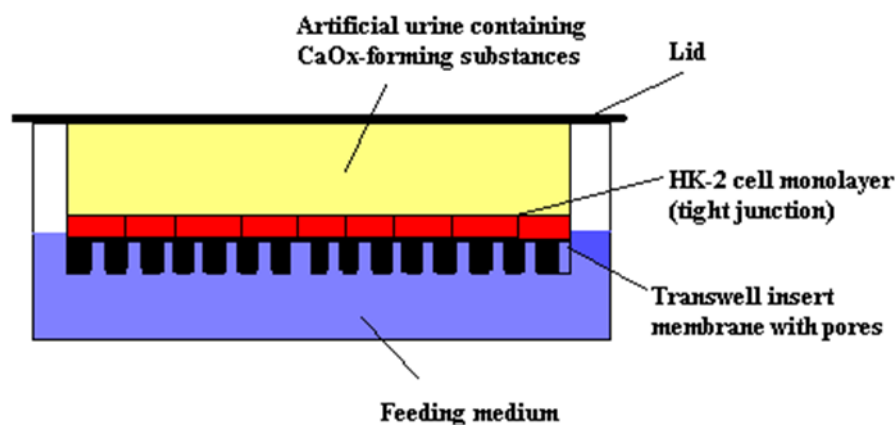
the human WT 9-12 cell line of distal and proximal cortical tubule (CRL-2833; ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC) with 10% fetal bovine serum (GIBCO, New York, NY) and in pre-coated culture flask with bovine collagen type I solution Sigma, St. Louis, MO) at 3.0 mg/ml concentration. Intact cell monolayer were established on permeable polycarbonate transwell

microporous filter membranes (insert growth area 1.12 cm², 0.4 μm pore size)(Coster® Corning, New York, NY) according to the standard protocol. The two compartments (Fig. 3) of the culture system (apical and basal layers) were then separated by the intact monolayer reaching TEER approximately at 300 ohm•cm². The

integrity of cell monolayer was determined using Epithelial Tissue Voltammeter (World Precision Instruments, Hong Kong, China) and the formation of tight junction was further confirmed by assessing the transepithelial flux of fluorescein isothiocyanate (FITC)-dextran (10 KDa; Sigma) from the apical side to the basal side.

Figure 3:

A two compartment trans-well culture system with tight junctions⁸. Apical and Basal sides of the cell are separated and the medium can be collected for further studies. HK-2 cells were replaced with WT 9-12 cell line.



Artificial urine (AU) was prepared as previously described⁸. Artificial urine was prepared daily. Analytical grade chemicals were dissolved in Milli-Q® water and pH was adjusted to 6.0 with hydrochloric acid. The final concentration of the major ions were calcium (6 mM), magnesium (3.0 mM), sodium (196 mM), potassium (82 mM), phosphate (23 mM), sulfate (20 mM), oxalate (1.2 mM) and citrate (2.2 mM). Urine was buffered with disodium phosphate (22 mM). Stock melamine solution (10 mM) was prepared by

dissolving 1.26 gm melamine (C₃H₆N₃, MW (126.1 g/mole), Sigma-Aldrich®) in 1 l Milli-Q water. Stock cyanuric acid solution (10 mM) was prepared by dissolving 1.2908 gm cyanuric acid (C₃H₃N₃O₃, MW (19.1 g/mole), Sigma-Aldrich) in 1 l Milli-Q water. Crystallization in supersaturated AU with respect to melamine cyanurate was observed microscopically and measured in terms of turbidity at 405 nm.

Cytotoxicity and cytokine panel expression

The concentrations of melamine, cyanurate and melamine/cyanurate that will cause cell toxicity by cell death were investigated on flat-bottom 96-well culture microplate, by measuring Lactate Dehydrogenase (LDH) released in media, in order to guide the concentrations to be used in subsequent experiments. In addition, the clinical relevant ratio for melamine and cyanurate, suggested as 99:1 was also tested⁹. On the transwell experiments, to allow formation of new crystals directly on the apical surface of WT 9-12 cells, equal amount (each 250 μ l) of appropriate dilutions of the melamine (50, 49.5, 25, 5 mmol/l concentrations) and cyanurate (50, 25, 5, 0.5 mmol/l concentrations) in AU were added separately into the insert well, and orbital shaking (10-minute) at 37°C was immediately followed by recording on the TECAN SPECTRA Fluor Plus microplate reader (TECAN, Grodig, Austria). Parameters tested were melamine to cyanurate ratios at 1:1 (50:50, 25:25, 5:5 mmol/l concentrations) and 99:1 (49.5:0.5 mmol/l concentration). For checking the cellular viability immediately after exposure, agitated cells were trypsinized and counted using the ViCell counter (Beckman Coulter Miami, FL) using the trypan blue exclusion method. In parallel experiments, AU solutions containing melamine/cyanurate crystals were removed after agitation and cells, washed thrice with phosphate buffered saline (PBS), replaced with complete media and incubated for 24 hours to study the repairing of cell monolayer. Such post-exposure incubation was aimed to allow sufficient time for cellular response

such as cell repair, gene expression, and cytokine secretion. At 24 hours, apical and basal media were collected for measurement for cytokine panel expression using eBioscience FlowCytomix T-helper (Th)1/Th2 11-plex and chemotaxis 6-plex assays (Bender MedSystems, Austria) with Cytomics FC500 Flow cytometer (Beckman Coulter, Miami) equipped with CXP software version 2.2. Cell nuclei were extracted for quantification of oxidative DNA damage using the highly sensitive 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA kit (Japan Institute for Control of Ageing, Shizuoka, Japan).

Human genome 44K gene expression microarray

Similarly to previous study with CaOx urolithiasis which constructed a expression profiling using cDNA microarray¹⁰, we also investigate the gene expression when the cultured cells were exposed to melamine/cyanurate under normal physiological conditions and high levels using the 44K Whole Human Genome Oligo microarray on Agilent genomic platform. Total RNA of harvested cells were extracted by using RNeasy Mini kit (Qiagen, USA). The quality of RNA was assessed in terms of quantity (absorbance at 260 nm), purity (A_{260}/A_{280} ratio of 1.9-2.1) and integrity (ratio of 28S rRNA to 18S RNA at 2:1 by using Agilent 2100 Bioanalyzer). All purified RNA samples were stored at -80°C until assay. Four conditions with melamine, cyanurate alone and in mixture 50:50 and 49.5:0.5 (melamine:cyanurate) were tested

against the AU control. The differentially regulated genes were then grouped into clusters based on their expression profiles and correlated with the cytokine expression panel work-up.

Statistical analysis

All assays were performed in duplicate for reproducibility. Differences between means were determined using ANOVA to compare mean differences with $p < 0.05$ considered statistically significant. All significant ANOVA test results were analyzed by the Dunnett multiple comparisons post-test. (GraphPad Prism Version 6.0 for Windows, GraphPad Software, San Diego, CA).

RESULTS

Patient Case studies #1 and #2

The clinical approach to hydrate and alkalinize the urine had been used as the first-line of therapy for the children affected by melamine “stones”. The laboratory findings on both the cases were not remarkable or distinguishing as regards to clinical presentation. Subsequent stone analysis by well renowned stone laboratory in Berlin, Germany confirmed the stones to be 100% uric acid crystals by X-ray Diffraction, FT-Infra-red spectroscopy and chemical methods.

Cell damage induced by melamine and cyanurate crystallization

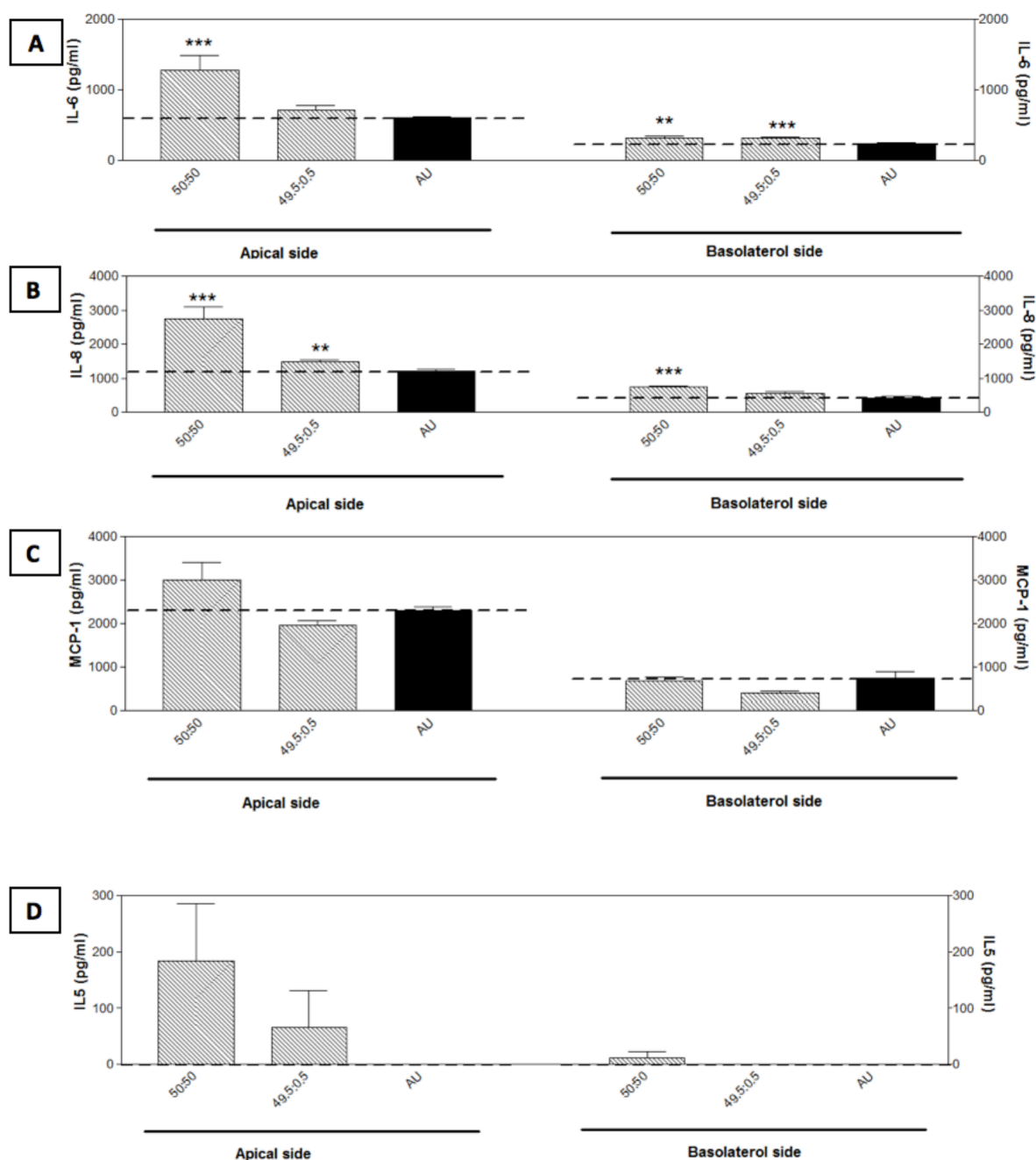
Direct cytotoxic effects, as assessed by the release of LDH, was demonstrated by a mixture of melamine and cyanurate in a concentration-dependent manner, but neither by melamine nor cyanurate alone (not shown). Oxidative stress (measured as oxidative DNA damage) was significantly ($P < 0.01$) induced by melamine/cyanurate at 1:1 ratio, by 1.13 $\mu\text{g/ml}$ 8-OHdG increase when compared with the AU control. However, such effect was not found in the culture with melamine/cyanurate at 99:1 ratio.

Influence of melamine and cyanurate crystallization on cytokine secretions

Sixteen Th1/Th2 cytokines and chemokines were tested and baseline levels of IL-6, IL-8 and (monocyte chemotactic protein) MCP-1 were detected in the harvested media of the cultures at 24 hours, and higher levels were measured at the apical side. The secretion of IL-6, IL-8 and MCP-1 were increased by the melamine/cyanurate crystals in parallel with their cytotoxic effects (Figure 4A-C). IL-5 was not detected in the control media, however, it was stimulated by the crystals (Figure 4D).

Figure 4:

The elevation of cytokines (A) IL-6, (B) IL-8, (C) MCP-1 and (D) IL-5 released in harvested media of cultured cells as stimulated by melamine/cyanurate mixture at 1:1 ratio (50mmol/l:50mmol/l) and 99:1 ratio (49.5mmol:0.5mmol/l), at 24 hours post exposure (** $P < 0.01$; *** $P < 0.001$)



Influence of melamine and cyanurate crystallization on the gene expression microarray

Over 7000 genes were up- or down-regulated in two-fold magnitude in the cultured cells by either the 1:1 or 99:1 (clinical relevant) ratio of melamine to cyanurate. Cluster analysis showed that genes involved in inflammation, calcium-gated channels and zinc finger proteins were mainly affected (not shown). Genes for butyrophilin-like, claudin 19 and voltage-dependent calcium channel variant

were remarkably down-regulated by the clinically relevant concentration ratio of 99:1, in contrast to other genes for leukocyte-associated immunoglobulin-like receptor, killer cell lectin-like receptor and synaptogyrin 1 were down-regulated by the 99:1 ratio (Table 1). However, up-regulation of certain cytokine genes including IL2, IL5, IL6, IL8 and chemokine-like factor were up-regulated by both ratios (Table 1), which correlated with the cytokine protein secretion examined in harvested media of culture and cytotoxic effects.

Table 1: Expression of interleukins / chemokines genes and their receptors caused by melamine:cyanurate at ratio 1:1 or 99:1 as compared with the AU control.

| Probe Name | Description | 50:50 | 49.5:0.5 | Melamine | Cyanurate |
|-------------------|---|-----------------------|---------------------------|-----------------|------------------|
| A_23_P501713 | Homo sapiens interleukin 1 family, member 10 (theta) (IL1F10), transcript variant 1, mRNA [NM_032556] | 1.6 down | 2 Down | 3.1 down | 2 down |
| A_23_P79398 | Homo sapiens interleukin 1 receptor, type II (IL1R2), transcript variant 1, mRNA [NM_004633] | 4.2 down | 3.1 down | 4.6 down | 3.5 down |
| A_23_P30115 | Homo sapiens interleukin 2 (IL2), mRNA [NM_000586] | 8 up | 9 up | 9 up | 9 up |

| | | | | | |
|--------------|---|-------------------------|-------------------------|-----------|-----------|
| A_24_P209047 | Homo sapiens interleukin 5 (colony-stimulating factor, eosinophil) (IL5), mRNA [NM_000879] | 3.2 up | 3.4 up | 3.6 up | 2.4 up |
| A_24_P935033 | Homo sapiens alternatively spliced interleukin-6 receptor beta chain mRNA, partial cds. [U58146] | 3.9 up | 4.3 up | 2.8 up | 2.7 up |
| A_32_P87013 | Homo sapiens interleukin 8 (IL8), mRNA [NM_000584] | 22 up | 13 up | 14 up | 25 up |
| A_24_P52733 | Homo sapiens interleukin 10 receptor, alpha (IL10RA), mRNA [NM_001558] | 1.6 up | 1.6 up | 1.2 up | 1.9 up |
| A_23_P7560 | Homo sapiens interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40) (IL12B), mRNA [NM_002187] | 1.1 up | 2.3 up | 1.1 up | 2.5 up |
| A_23_P61057 | Homo sapiens interleukin 16 (lymphocyte chemoattractant factor) (IL16), transcript variant 1, mRNA [NM_004513] | 3 up | 3 up | 3.5 up | 3.6 up |

| | | | | | |
|--------------|--|----|----|----|----|
| A_24_P215804 | Homo sapiens | 3 | 14 | 14 | 6 |
| | chemokine-like factor (CKLF), transcript variant 1, mRNA [NM_016951] | up | up | up | Up |

DISCUSSION

Milk products were mainly contaminated with melamine, and a by-product contaminant - cyanurate - with less than 1% , and this ratio of 99:1 is taken to be clinically relevant in our studies⁹. Unlike CaOx crystals which have been shown to be actively taken up by renal tubular cells^{8,11}, melamine crystals caused physical damage to the cells and did not have any appearance of adherence to the cell surface - a necessary event before endocytosis occurs. LDH is a classic marker for cell-damage and subsequent oxidative stress on cells can be seen with melamine cyanurate present at 1:1 only and not at clinically relevant 99:1 ratios. Such cellular damage was also seen on human erythrocytes¹².

Significant increase of IL-5, -6, -8 and MCP-1, suggests the cellular response to renal cell injury and promoting the recruitment of host immune-competent cells such as neutrophils and monocytes. The presence of IL-5 and -6 also suggested a shift to Th2, which favors the humoral type of immune response. There have been reports of melamine inducing antibodies¹³

and being able to raise antibodies in experimental models¹⁴. Hence, for cells to release mediators to support / enhance humoral type of immune response is consistent with the in-vivo studies in humans and animals. The overall microenvironment suggested that the crystals caused physical cell injury on the tubular cells to trigger pro-inflammatory reactions. The increase of cytokine levels at both apical and basolateral sides also suggested the interference of tight junctions on the cell monolayer.

We report the gene expression microarray using whole genome scanning (Human genome 44K) when melamine/cyanurate is presented to the cells in culture. Invariably, during such studies using global scanning, there will be expression of several thousands of genes, cluster analysis of the top 100 genes expression showed that gene clusters involved in inflammation, calcium-gated channels and zinc finger protein were the major clusters that were expressed. This is similar to the in-vivo animal model of global gene expression conducted on rats with CaOx urolithiasis¹⁵, However, in our model, the magnitude of up/down regulation was

several times higher and the genes shown in that study were distinctly different from those observed by us in the current study. This can be hypothesized that injury and uptake of CaOx urolithiasis is distinctly separate from melamine induced crystallization. The gene expression observed here, does give a snapshot of the possible types of cell responses to injury by melamine - mainly the inflammatory and Th2-humoral type of immune response. This is corroborated with the findings of cytokines profile secreted in the culture media. As revealed in other studies with human kidney epithelial cells, genes for inducible cytokine subfamily were commonly involved in the crystal-induced cellular injury events¹⁰.

Case studies and stone fragments analyzed indicated that the stones retained by the children were of pure uric acid. The latter did not get resolved through hydration and alkalization treatment. It has been reported by Dorne et al¹⁶ that uric acid is a frequently found with melamine / cyanurate crystals in animals and humans. Urine is supersaturated with ions and addition of more ions (melamine and/or cyanurate) with complementary epitaxial relationships would precipitate out such crystals and thus it is not surprising to find these in humans. The absence of finding any melamine or cyanurate in the stone fragments is promising, in that, such crystals were successfully eliminated from the renal system and no long-term damage or harm is expected.

In conclusion, melamine–crystal cell interaction was particularly different from other nephrolithiasis event (eg. CaOx and Calcium Phosphate (CaP)). Presence of clinically relevant levels of melamine brought about physical damage to cells with the concomitant release of cell injury markers, LDH and oxidative stress. It should be noted that the few stone samples recovered from patients, turned out to be uric acid samples. The release of cytokine markers for inflammation and humoral immune response were evident and this is correlated to that found in other studies. Overall, there was no evidence of long-term sustained damage of cells from melamine exposure, except of the physical effects. However, the incident created a number of public health issues worldwide^{17,18}. From our current studies, we can now have a better understanding of the cellular effects of melamine, which is particular important in understanding the clinical manifestations of melamine related problem and also establishing a clinical management plan.

ACKNOWLEDGEMENT AND FUNDING SOURCE

This project was funded by HKSAR Food and Health Bureau Grant M1-BS-07 awarded to Prof C.F. Ng at the Chinese University of Hong Kong. Prof Gernot Schubert of Vivanter Klinikum, Berlin, for his assistance in the analyses of the patient stone fragments.

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