Clinica Chimica Acta 430 (2014) 96–103

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Kidney stone distribution caused by melamine and cyanuric acid in rats

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ARTICLE INFO

Article history:
Received 25 December 2013
Accepted 3 January 2014
Available online 11 January 2014

Keywords:
Melamine and cyanuric acid
Acute toxicity
Renal crystals
Distribution

ABSTRACT

Background: Melamine (M), which is composed of multi-amine, has been used as a food additive to falsely increase protein contents. Furthermore, cyanuric acid (CA) is a derivative of melamine. It is known that these mixtures can cause renal toxicity.
Methods: The objective of this study was to investigate the possible target cells during acute renal toxicity of melamine and cyanuric acid (MCA) mixture crystals *in vivo*. Rats were provided with a lethal dose of MCA (1:1; 400 mg/kg) and observed after 0.5, 1, 3, 12, 24, and 48-h intervals.
Results: MCA caused degeneration/necrosis in the proximal tubules starting at 12 h and increased at 24 and 48 h. A small number of yellow-green crystals were observed in the dilated distal renal tubules at 48 h post-treatment. Ultrastructurally, pyknosis, mitochondrial vesicles, and cellular swelling were found in the proximal tubular cells at 0.5 h. Small needle-like crystals in the cytoplasm and large crystals in the lumen of tubules indicated physical damage to the renal cells.
Conclusion: These results clearly reveal that in the MCA-induced renal toxicity model, crystals are distributed to both the proximal and distal tubules in rats. The proximal tubular cells may be initially injured and subsequently block the distal tubules with MCA crystals during early acute intoxication.

1. Introduction

In 2004 and 2007, an outbreak of melamine and cyanuric acid contamination in dog and cat food products occurred, causing kidney failure and kidney stones [1–3]. In 2008, Chinese infant formula adulterated with melamine caused babies to develop kidney and bladder stones [4]. Melamine is composed of multiamine and is added to food to falsely increase its protein content [5]. A recent study showed that the melamine metabolite formed by microbial metabolism plays the crucial role in causing kidney damage in rats [6]. However, whether cyanuric acid is added to melamine or is a breakdown product of it is still unclear.

Prior to the incident, melamine, cyanuric acid, and other analogs were well investigated. The median lethal dose (LD_{50}) of melamine and cyanuric acid orally administered in rats was found to be 3161 and 7700 mg/kg body weight, respectively, indicating that these two substances are of low toxicity. Whatever the causes were of acute renal failure in dogs and cats in the 2004 and 2007 events, the combination of melamine and cyanuric acid appeared to be more toxic than each substance alone, and it increased renal toxicity substantially [7]. Renal failure is currently speculated to be a result of urine concentration caused by crystallization in kidneys, leading to tubular cell necrosis, regeneration, and expansion. After long-term damage, renal fibrosis and inflammatory cell infiltration occur [8]. Using rat models, renal crystal formation following the ingestion of a melamine–cyanuric acid mixture (1:1) was investigated to gain insight into MCA-induced renal toxicity [8].

Kidney stone formation has been related to supersaturated-urine. A continuing supersaturated urine concentration, an increase in crystallization, and crystal nuclei formation lead to the gathering of crystals to form stones over time [9]. Crystal deposition in renal tubule causes tubular expansion, resulting in chronic inflammation, chemotaxis of inflammatory cells with inflammatory cytokine secretion and elevation of oxidative stress. Previous studies have shown that the shape of melamine–cyanurate crystals is rounded *in vivo* and needle-like *in vitro*, and that the crystal shape was unaffected by the pH level [10]. As with other common kidney stones, melamine and cyanuric acid-related renal stones are similar to the stone formation theory. Whether crystalline and tubular obstructions play the main role in renal failure has yet to be determined. No obstructive nephropathic symptoms

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http://dx.doi.org/10.1016/j.cca.2014.01.003

such as hydronephrosis or cystic kidney have been found with renal failure as a result of a melamine and cyanuric acid combination. Melamine mixed with cyanuric acid could obviously have strong toxic effects, but other mechanisms related to the toxic effects still require further study [11,12].

This study was conducted to further investigate the target cells of acute nephrotoxicity in MCA-intoxicated rats and elucidate the possible pathogenesis at different time points within 48 h.

2. Materials and methods

2.1. Chemicals

Melamine (M, 2, 4, 6-triamino-1, 3, 5-triazine) was obtained from Sigma (MO, USA) and cyanuric acid (CA, s-triazine-2, 4, 6-triol) was obtained from Merck (Darmstadt, Germany). The chemical purity of the commercially obtained materials was 99% for melamine and 98% for cyanuric acid.

2.2. Animals

Male Sprague–Dawley rats (six in the control group and five in the experimental group, 4 weeks old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The rats weighed 140–160 g at the time of the study. Animals were housed individually in cages and given lab chow *ad libitum* (Purina 5001, St. Louis, MO, USA). The temperature of the room housing the animals was maintained at 22–25 °C, the relative humidity at 50–70%, and photoperiods were 12 h/day. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung-Hsing University (IACUC: 98-83).

Experiments were conducted to investigate the renal toxicity of MCA on target cells. Therefore, a 400 mg/kg lethal dose of MCA was selected, based on previous studies [13]. Melamine and cyanuric acid have poor water solubility at 3230 mg/L and 2000 mg/L, respectively [5]. Therefore, propylene glycol (PEG-200) was used as the delivery vehicle. A single dose of MCA (400 mg/kg/bw) was orally gavaged. The administered volume of MCA was 10 mL/kg body weight. For the time-course study, rats were observed in the six time courses of 0.5, 1, 3, 12, 24, and 48 h intervals. For reducing the use of laboratory animals, only one control group (48 h) was designed and sacrificed at the end of experiment.

2.3. Hematological, biochemical and urinary examinations

The animals were anesthetized using 3% isoflurane inhalation, and blood was drawn from the abdominal aorta artery at the end of the experiment. Blood was collected separately in anticoagulant EDTA tubes and serum separator tubes (Vacationer, Franklin Lakes, NJ, USA). Whole blood from the anticoagulant tubes was stored at 4 °C, and parameters of WBC, RBC, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, hemoglobin, and platelets were analyzed on a blood cell counter (Sysmex K-4500, Toa Medical Electronics Co., Kobe, Japan). Blood smears were stained with Weigert's Iron Hematoxylin Stain Kit (AJP Scientific Inc., NJ, USA) for cellular differentiation. The percentages (%) of lymphocytes, neutrophils, and monocytes in leukocytes were calculated. Blood urea nitrogen (BUN) and creatinine were analyzed by enzymatic methods using an automatic analyzer (Chiron Diagnostics Corporation, OH, USA). Also, serum calcium, magnesium, phosphate, and potassium were measured using an AVL Model 9130 automatic electrolyte analyzer (AVL Scientific Corporation).

2.4. Urinary examination

Rat urine samples were collected at 0.5, 1, 3, 12, 24 and 48 h intervals. Urine sediments were centrifuged, and the total number of leukocytes, RBC, epithelial cells, and crystals were evaluated under light microscope (BX-51, Olympus, Tokyo, Japan).

2.5. Histopathological examination

At necropsy, the kidneys were weighed and sectioned transversely. The middle section of the other half of each kidney was fixed in 10% neutral buffered formaldehyde. The formaldehyde-fixed tissues were trimmed, embedded in paraffin, sectioned (Leica RM 2145, Nussloch, Germany), and stained with the hematoxylin and eosin (H&E) method, then evaluated under light microscope for histopathology. For semi-quantitative grading of renal toxicity, lesion severity was graded using the criteria developed by Shackelford et al. (2002) [14] as follows: 1 = minimal (<10%); 2 = slight (11–25%); 3 = moderate (26–50%); 4 = moderate/severe (51–75%); and 5 = severe/high (76–100%).

2.6. Scanning electron microscope observation

The remaining part of each kidney was cut into 0.5 × 0.5 × 0.5 mm pieces and fixed in 2.5% glutaraldehyde solution for examination under a scanning electron microscope. The tissue was dehydrated with serial alcohol. The samples were fixed on copper containing carbon glue and put into the Critical Point Dryer (CPD). The surface of the sample was coated with gold, and morphological changes of the MCA crystals in kidneys were observed using scanning electron microscopy. Crystals were further detected by SEM-EDS.

2.7. Transmission electron microscope examination

After soaking in neutral phosphate buffer, the kidney tissues were cut and post-fixed in 1% osmium tetroxide for 3 h. The tissues were dehydrated with serial alcohol in order to dry completely and embedded in LR White acrylic resin. The embedded samples were sliced in thicknesses of 90 nm. The slices were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (1200EX, JEOL, Tokyo, Japan).

2.8. Statistical analysis

Data are expressed as means ± SD. A statistical difference test was performed between the control and treated groups by Student's *t*-test. Differences are regarded as significant at $P < 0.05$.

3. Results

3.1. Clinical observation

No obvious clinical symptoms were found in the control group or in the 0.5, 1, 3, and 12 h MCA treated groups. However, the 24 and 48 h MCA treated rats became anorexic, weak, depressed, and developed a

Table 1
White blood cell differentiation alterations of rats gavaged with 400 mg/kg melamine and cyanuric acid after 0.5, 1, 3, 12, 24 and 48 h intervals.

Time (h)	WBC (10^3/μL)	Lymphocyte (%)	Neutrophil (%)		Monocyte (%)
			Band	Segmented	
Control	6.5 ± 1.8	92.8 ± 2.5	0.0 ± 0.0	6.0 ± 2.1	1.0 ± 0.6
0.5	10.6 ± 2.2*	91.6 ± 2.5	0.0 ± 0.0	6.2 ± 1.7	2.0 ± 0.6*
1	6.9 ± 0.7	88.8 ± 3.7	0.4 ± 0.4	7.2 ± 2.0	3.6 ± 2.2*
3	8.4 ± 3.4	86.6 ± 4.6*	0.8 ± 0.9	11.2 ± 4.7	1.4 ± 1.5
12	12.0 ± 1.5*	64.0 ± 10.2*	3.3 ± 1.4*	30.3 ± 0.9*	2.3 ± 0.9*
24	8.3 ± 2.6	72.2 ± 5.9*	2.4 ± 1.5*	22.8 ± 5.3*	2.6 ± 1.0*
48	5.5 ± 2.1	65.2 ± 14.1*	6.4 ± 4.3*	28.0 ± 11.2*	0.4 ± 0.4

Data are expressed as the mean ± SD (control $n = 6$, treatment $n = 5$).
* Significant difference between the control and treated groups at $P < 0.05$.