Effects of water uptake on melamine renal stone formation in mice

Jiao Peng, Daxu Li, Yee Kwan Chan, Yan Chen, Jonathan R. Lamb, Paul K.H. Tam and Hani El-Nezami

1Department of Surgery, LKS Faculty of Medicine, the University of Hong Kong, Hong Kong, China, 2School of Biological Sciences, the University of Hong Kong, Hong Kong, China and 3Division of Cell and Molecular Biology, Faculty of Natural Sciences, Imperial College London, London, UK

Correspondence and offprint requests to: Yan Chen; E-mail: ychenc@hku.hk

Abstract

Background. Melamine-tainted food can induce kidney stones both in humans and animals and in domestic animals, severe cases caused acute kidney failure and death. Although increasing water intake can ameliorate kidney stone formation, its effect on melamine (Mel)-induced kidney stones has not been studied.

Methods. We have analysed the effect of restricted ingestion of drinking water on melamine stone formation in mice. They were given melamine and cyanuric acid orally and received drinking water either freely or for a restricted time. Kidney stone formation and renal function were monitored.

Results. Mice receiving drinking water for a restricted 10-h period initially lost body weight, which returned to normal within 2 days. No other abnormalities were observed. Ingestion of melamine alone failed to induce kidney stones even under conditions of restricted drinking water. In mice treated with melamine together with cyanuric acid for 3 days, no renal stones were formed when the supply of drinking was normal. However, when drinking water was limited, stone formation was observed and accompanied by high levels of serum urea and creatinine. An increase in urine haemoglobin and glucose levels was also found. The administration resulted in up-regulated tissue osteopontin, kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin messenger RNA expression and macrophage infiltration.

Conclusions. Our results indicate the importance of water intake in the formation of melamine-induced renal stone formation in the mouse and provide new information on the mechanisms of melamine stone formation.

Keywords: melamine renal stone; mouse; water ingestion; osteopontin; macrophage

Introduction

Melamine-tainted milk affected tens of thousands of Chinese children during 2008 to 2009 causing altered renal function due to the formation of kidney stones [1]. Acute kidney failure was reported in cats and dogs fed melamine-tainted pet food, which eventually caused their death [2]. Effective treatments have included liquid supply and alkalization of urine [3], and only in severe cases, was surgical intervention such as ultrasound-guided extracorporeal shockwave lithotripsy required. The ingestion of water provides an effective treatment by flushing out renal stones but whether or not water deprivation also plays a role in melamine stone formation has not been studied. Cyanuric acid was identified as a component in the pet food that enhanced toxicity of melamine and it was concluded that melamine together with cyanuric acid could effectively induce acute kidney failure. In contrast in human studies following ingestion of melamine alone, there was no formation of renal stones [2] and when stone formation occurred, it was unrelated to cyanuric acid [1, 4].

Kidney stones are a common pathology in humans and animals. Companion animals are often used in the study of renal stone formation [5] but few studies have been performed in mice. Studies in mice revealed that increased expression of osteopontin (OPN) reduces renal stone formation induced by oxalate [6] and when OPN knockout
mice were fed with ethylene glycol (an oxalate precursor), they developed kidney stones [6].

Here, we have investigated the effects of restricted ingestion of drinking water on melamine-induced renal stone formation in mice. In addition to the presence of renal stones, serum levels of creatinine and urea, OPN expression and macrophage infiltration were monitored. The biomarkers of tubular damage, such as kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) [7] were measured. Our data indicated that restriction of drinking water contributes to the formation of renal stones in melamine- and cyanuric acid-treated mice.

Materials and methods

Animals and reagents

Inbred C57BL/6 mice (6–8 weeks old and weighing 20–30 g) were used in the experiments. The experimental protocol was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Melanine, cyanuric acid and ethylene glycol were purchased from Sigma (Sigma-Aldrich, St Louis, MO).

Experimental design and animal administration

The mice were separated into four different groups: normal drinking water (Nor-H2O) control group—the animals were given normal access to water; restricted drinking water (Alt-H2O) control group—where water was supplied only during day time (Figure 1A); the melamine (Mel) group—received the compound (400 mg/kg/day) and restricted drinking water; in the melanine and cyanuric acid (Mel + CA) group—the mice were treated with both melamine and cyanuric acid (40 mg/kg/day) and drinking water was restricted. Melamine and melamine with cyanuric acid were suspended in sodium carboxymethyl cellulose before oral delivery. To evaluate the effect of cyanuric acid (CA), the compound (40 mg/kg/day) was administered with a normal or restricted water supply.

Histology and immunohistochemical analysis

The liver and kidneys were removed and fixed in 10% formalin and then embedded in paraffin or stored at −20°C. The weight of the mice was measured daily (Figure 1). Mice were separated into four different groups: normal drinking water (Nor-H2O) control group—the animals were given normal access to water; restricted drinking water (Alt-H2O) control group—where water was supplied only during day time (Figure 1A); the melamine (Mel) group—received the compound (400 mg/kg/day) and restricted drinking water; in the melanine and cyanuric acid (Mel + CA) group—the mice were treated with both melamine and cyanuric acid (40 mg/kg/day) and drinking water was restricted. Melamine and melamine with cyanuric acid were suspended in sodium carboxymethyl cellulose before oral delivery. To evaluate the effect of cyanuric acid (CA), the compound (40 mg/kg/day) was administered with a normal or restricted water supply.

Blood and urine chemical analysis

Serum was collected and stored at −80°C until use. Blood chemical analysis related to kidney and liver function were performed in Pathlab, Medical Laboratories LTD, Hong Kong. Mouse urine was collected on Days 0 and 3 of the experiments and the estimation of kidney function was performed with test paper purchased from Medi-Test Combi 5S (MA-SHERY-NAGEL GmbH & Co. Germany).

Statistical analysis

Data are presented as mean ± SD and at least three mice were used in each group. Sections (>10) were cut and analysed for each sample. Student’s t-test was used when comparing two sets of data and one-way analysis of variance was used when three or more sets of data were analysed. The statistical analysis was performed by using Prism 5.0 software (GraphPad Software, Inc, CA) and P < 0.05 was considered statistically significant.

Results

Effect of restricted drinking water ingestion and exposure to melamine/cyanuric acid on mouse body weight

The weight of the mice was measured daily (Figure 1). Mice receiving ad libitum amounts of drinking water, in the Nor-H2O group, had no change in weight within 3 days. However, there was a slight reduction in body weight (3.44%, P > 0.05) at Day 1, which recovered by Day 2 and was normal at Day 3 in those mice receiving a restricted supply of drinking water (Alt-H2O) as well as in the Mel group. In the melamine plus cyanuric acid (Mel + CA) group, the weight loss began at Day 1 and was further significantly reduced at Days 2 and 3 (6.35% compared to initial weight, P < 0.05). Thus, the combined administration has systemic effects on the health of the mice.

Melamine with cyanuric acid induces renal stone formation in mice with restricted access to drinking water

Compared to the Nor-H2O group, the Alt-H2O and Mel groups displayed no obvious differences in gross behaviour. In the Mel+CA group, mice were lethargic and anorexic. At necropsy, both liver and kidneys were pale. Histological analysis revealed that there were melamine stones in the kidney with single or multiple crystals in the cortical area and enlargement of tubules (Figure 2A). Enlarged hepatocytes and oedema were observed in marginal region in Mel + CA. In kidney paraffin sections, stones were mainly situated in enlarged tubules in cortical area, but some were also present in the collecting ducts. No stones were found in the glomerulus but shrinkage of glomeruli was observed in some cortical regions (Figure 2B).
The solubility of melamine-induced stones was examined using H$_2$O, ethanol (100%), formalin (10% vol/vol), Na$_2$HCO$_3$ (1M), HCl (1N) or NaOH (1N) on the frozen sections. The results revealed that treating the melamine stones with HCl solution for 30 min completely dissolved them, whereas NaOH resulted only in partial solubilization (~50%; Figure 2C).

Changes in serum and urine biomarkers of renal and hepatic function

Comparing the Nor-H$_2$O, Alt-H$_2$O and Mel alone groups, there was no evidence of altered renal function with respect to urea and creatinine levels (Figure 3A). When treated with melamine/cyanuric acid, urea levels were significantly increased to 30.7 ± 10.5 mM (normal level 6.4–10.4 mM), and creatinine was increased to 46.7 ± 15.2 µM (normal level 18–71 µM), P < 0.05 (n = 3 in each group) suggesting functional damage of the kidneys had occurred. In the liver, serum glutamic oxaloacetic transaminase / Aspartate transaminase levels were slightly increased and no change was detected in bilirubin in the melamine or Mel + CA-treated groups indicating no obvious alteration in liver function (Figure 3A). Compared to the Nor-H$_2$O and Alt-H$_2$O groups, animals treated with melamine alone showed an insignificant increase in erythrocytes in the urine; however, with melamine/cyanuric acid administration, they were significantly increased (P < 0.05 n = 3). Similarly, urine glucose was slightly increased in the Mel group at Day 3 compared to control group, while in the Mel + CA administration group, a significant increase was observed (P < 0.01, n = 3). Urinary pH in Nor-H$_2$O and Alt-H$_2$O groups was normal but in some of the Mel and Mel + CA-treated mice, the pH < 4.5 (Figure 3B).
Renal macrophage infiltration in melamine and melamine-/cyanuric acid-treated mice

Erythrocytes and glucose in urine of the Mel + CA group suggested that renal inflammation had been induced. Immunostaining with rat anti-mouse F4/80 antibody revealed that macrophages in the kidney were present at 3-fold higher numbers in the melamine group compared to controls (P < 0.001). Mel + CA-treated mice had the highest number of infiltrating macrophages which were localized mainly in the tubular area. Macrophages were also present in the glomerular region in the experimental groups treated with melamine or melamine/cyanuric acid (Figure 4). When the water was given together with Mel + CA, macrophage numbers were significantly reduced (P < 0.001).

Fig. 3. Blood and urine biomarker analysis of melamine and melamine-/cyanuric acid-treated mice. (A) Kidney and liver function is assessed by the analysis of creatinine, urea, SGOT/ASA and bilirubin levels. Blood was collected at the end of the experiments and analysed by Pathlab, Hong Kong. The results were compared. *P < 0.05 when compared with Nor-H2O group, n = 3 in each group. (B) Mice urine was collected before and at end of experiments. The total volume was measured. The urine haemoglobin, glucose and pH were measured with Medi-Test Combi 5S and the data were compared. *P < 0.05, **P < 0.01 compared with Nor-H2O group, n = 3 in each group.

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Renal expression of OPN and NGAL in response to melamine administration

OPN is a key regulatory factor in oxalate stone formation [6] and a macrophage chemoattractant [8]. As macrophage infiltration was observed in affected kidneys after administration, expression of OPN was examined by qRT–PCR. Our results revealed that the restricted drinking water protocol slightly reduced OPN expression but no obvious effect of melamine or cyanuric acid was observed compared to normal water supply. However, in Mel + CA group, the OPN mRNA was increased 5-fold compared to the control group (P < 0.001; Figure 5). Thus, OPN may play a role in melamine stone formation in this model.

NGAL has been used as one of the biomarkers in acute kidney injury [7]; therefore, we investigated the effects of melamine on its expression. The expression pattern of KIM-1- and NGAL-specific transcripts was similar to that of OPN, namely that it was only significantly increased (>50-fold) in the Mel + CA group receiving a restricted water supply and no obvious alternations were observed in all other treatment groups (Figure 5).

Discussion

The effects of melamine and cyanuric acid uptake on kidney stone formation have been reported in fish, cats, dogs and pigs [5, 9] but not in mice. Melamine has a short half-life in tissues and therefore, we investigated if prolonged retention of melamine within tissues caused by restricted drinking water intake would increase renal stone formation in mice. In fact, our procedure has only restricted time of water supply during food intake (together with melamine and cyanuric acid) at night, the mice might have compensated by overdrinking of water the next morning which was supported by not much difference being found in urine volume at Days 0 and 3 (Figure 3B).

This study and the others indicate that melamine administration alone without cyanuric acid cannot induce renal stone formation in mice.
stone formation in experimental animals [9, 10]. When melamine and cyanuric acid were administered together for 3 days, melamine crystals were found in the kidney, which was accompanied by acute kidney failure. However, this phenomenon only occurred when access to drinking water was limited. Under conditions of drinking water, ad libitum crystals were found only very occasionally, no dilated tubules were observed in tissue sections and the mice had no symptoms of kidney failure. The half-life of melamine in the blood is 2.7 h and it is cleared mainly through the renal system [11], thus it is possible that by restricting the availability of drinking water, melamine and cyanuric acid were retained longer with a high concentration in the kidney allowing 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 was occurring rapidly. Our results partially explained that the melamine stone mostly caused acute kidney failure in patients and animal studies. It has been shown that the melamine can be degraded to cyanuric acid in vivo [12, 13]; however, the administration with melamine alone resulted in no stone formation suggesting that the concentration was not high enough to form the melamine–cyanuric acid crystal, which is confirmed by our observation that low concentrations of cyanuric acid with the same concentration of melamine produced less number of stones (data not shown). The detailed measurement of cyanuric acid with liquid chromatography tandem mass spectrometry may help to clarify the amount of cyanuric acid produced in mice.

Renal macrophage inflammatory cell filtration is an important consequence of oxalate stone formation [14]. The self assembly of melamine and cyanuric acid to form crystals occurs in vitro and may also do so in vivo [9] which would recruit and activate macrophages and consequently contribute to kidney damage. In the melamine-/cyanuric acid-treated mice with renal failure macrophages had infiltrated both the renal tubules and glomerular region implying that they cause kidney damage as indicated by haemoglobin and glucose in the urine. The ingestion of melamine alone increased macrophage infiltration concomitant with slightly augmented levels of haemoglobin and glucose in the urine. However, no tissue damage was found as indicated by KIM-1 and NGAL expression (Figure 5), the biomarkers of acute kidney injury [7], suggesting that leakage of haemoglobin may be due to the acute toxic effects on the kidney epithelial cells. In response to melamine plus cyanuric acid, the recruited macrophages might be activated by melamine crystals which could further exacerbates the injury and in this experimental group, KIM-1 and NGAL expression was greatly augmented. It has been shown that the macrophages are more sensitive than other cell types to the cytolytic effects of melamine and melamine/ cyanuric acid [15], therefore resident renal macrophages may contribute to the initiation of kidney damage. The depletion of renal macrophages will help to identify the function of macrophages in the damage caused by melamine stone formation.

OPN prevents oxalate formation in mice [6] but it is also pro-inflammatory and can enhance the activity of MCP-1, a macrophage chemoattractant [8]. The increase of OPN expression in the melamine/cyanuric acid group may in part account for increased macrophage infiltration. However, in mice treated with melamine alone as well as cyanuric acid with limited access to drinking water OPN was not up-regulation, thus melamine and cyanuric acid has no apparent direct effect on OPN expression. It is possible that up-regulation of OPN is due to the mechanical effects of the crystals. The rat was one of the small animals used in the melamine stone study and the stone formation can be observed without the restricted water process [16]. The phenomenon was similar to that of an ethylene glycol-induced oxalate renal stone that, with the administration in the rat can easily induce stones, but only in OPN−/− mice can the stone formation be observed further suggesting that OPN may play an important role in melamine plus cyanuric acid-induced stone formation. Studies of oxalate and melamine stone formation in rats and mice suggested that high expression of OPN has beneficial effects in preventing stone formation. However, the same procedure using OPN−/− mice will provide definite information in the role of OPN in melamine stone formation.

In conclusion, our study reveals that restricted intake of drinking water increases the potential of melamine and cyanuric acid to cause renal stone formation in mice. This may prove to be a useful model to study the mechanisms of renal stone formation and access potential therapeutics.

Acknowledgements. The authors thank Dr Sydney Tang, Dr Lawrence CL Lan, Dr Mingfu Wang, Prof Frederick Leung, Prof Feng Chen and Prof Allan SY Lau, the University of Hong Kong for help with data analysis and critical discussion of the results. The authors have no competing interests that might be perceived to influence the results and discussion reported in this paper. This work was supported by Food and Health Bureau, The Government of the Hong Kong (grant number MI-BS-16).

Conflict of interest statement. None declared.

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Received for publication: 30.6.11; Accepted in revised form: 29.8.11