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ORIGINAL PAPER

Renal tubular epithelial cell injury, apoptosis and inflammation are involved in melamine-related kidney stone formation

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Abstract The objective of this study is to understand pathogenesis of melamine-related kidney stone formation. We investigated the characterization of renal tubular cell under exposure to a mixture of melamine and cyanuric acid in vivo. Male Sprague-Dawley rats were separated into two experimental groups. Treatment group was administered daily with a standard commercial diet mixing with melamine and cyanuric acid, and control group was given a normal diet. Rat kidney specimens were stained with hematoxylin/eosin and the crystals were examined using a polarizing microscope. Renal tubular epithelial cells were observed by transmission electron microscopy. Semiquantitative RT-PCR assay was performed to determine monocyte chemoattractant protein-1 (MCP-1) mRNA expression, a protein in response to various proinflammatory stimuli. Apoptotic cells were examined by TUNEL assay. Melamine-associated crystals formed in glomerulus and wide renal tubule segment including proximal convoluted renal tubules, distal convoluted renal tubules, the limb loops of Henle and medullary collecting ducts in the cortex and medulla. Light microscopy results showed that the crystals lead to tubular lumen dilatation and tubular epithelial cell necrosis. It was observed that nucleus of renal tubular epithelial cells became irregular outlines and condensed, lysosomal-related structures increased, and integrity of renal tubule was deficient under electron microscopy. Apoptotic cells were noted widely in cortex and medulla. MCP-1 mRNA expression was significantly increased in the melamine and cyanuric acid-administrated group. Renal tubular epithelial cell injury, apoptosis and inflammation are involved in melamine-related kidney stone formation. Our findings are important for understanding pathogenesis of melamine-related kidney stone formation and estimating its clinical prognosis.

Keywords Melamine · Kidney stone · Cell injury · Apoptosis · Inflammation

Abbreviations

MRCs	Melamine-related crystals
PCT	Proximal convoluted renal tubule
DCT	Distal convoluted renal tubules
MCD	Medullary collecting ducts
MCP-1	Monocyte chemoattractant protein-1
TUNEL	Terminal dideoxynucleotidetransferase-mediated X-dUTP nick end labeling
TEM	Transmission electron microscope

Introduction

Kidney stone is a complex multifactorial disease resulting from an interaction between the environmental and genetic

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factors. The incidence of kidney stone has been increasing over the half past century. The lifetime risk of kidney stones is currently at 6–12 % in North American and 3.8–9 % in Asian population [1, 2]. To our knowledge, common kidney stones are composed of calcium oxalate, uric acid, struvite and cystine. However, a new melamine-related kidney stone was recently detected in China, and melamine and cyanuric acid come into view as malefactor.

Melamine, a material for resins used in producing plastics, was known as a new risk of kidney stone formation. Melamine has high nonprotein nitrogen content, and was added to milk or animal feed to raise falsely the apparent protein content of these products. About 300,000 children have been affected by milk powder contaminated with melamine in the People's Republic of China. Several investigations have provided with substantial relation between exposure to melamine-containing milk powder (the highest concentration of 2,563 mg/kg) and nephrolithiasis in infants and children [3, 4]. Cyanuric acid is melamine's "partner-in-crime". The reason for the presence of cyanuric acid is unknown; it may also have been added intentionally or perhaps was a by-product of melamine synthesis [5]. Melamine and cyanuric acid alone can be rapidly excreted in the urine, and failed to cause kidney stone and acute renal toxicity [6, 7]; whereas, a mixture of melamine and cyanuric acid can form an insoluble molecular complex at pH 5.8 and form melamine-related crystals (MRCs) in renal tubules [8].

It is a complex process from crystals to stone formation for all of types of stone. Some studies found that renal stone formation depends not so much on forming crystals, but on interaction of crystals and renal tubular epithelial cells. Crystals could be excreted with urine flow and would not form stone unless it remains in the renal tubules. In general, renal tubules injury, dysfunction can promote crystal nucleation, aggregation and retention [9]. In addition, localized inflammation may also be associated with the deposition of various urinary crystals in the renal tubule during kidney stone formation [10]. Up to now, the pathogenesis of melamine-related kidney stone formation on renal tubule is still unclear. In the present study, we have generated the melamine-related kidney stone rat model successfully and found that the mixture of melamine and cyanuric acid may lead to renal tubular epithelial cells injury, apoptosis and inflammation.

Materials and methods

Animal models

Six-week-old male Sprague-Dawley rats weighing 100–120 g were obtained from VITAL RIVER (Beijing, CN). The rats were bred in the college animal facility and separated into two experimental groups, eight for each group. Treatment group were administered daily with a standard commercial diet mixing with melamine (250 mg/kg) and cyanuric acid (250 mg/kg), and control group was given a normal diet. Rats were housed in metabolic cages with a controlled temperature and given plain water. The rats were killed at fourth week and their kidneys were removed after sufficient reperfusion. The left kidneys were stored at −80 °C for further molecular biological studies, and the right kidneys were fixed overnight at 4 °C in 4 % paraformaldehyde for histological studies. The experiment was reviewed and approved by the Shenyang Medical College Animal Committee.

Histological studies

Rat kidney specimens were embedded in paraffin. Cross-sections cut at a thickness of 5 μm were stained with hematoxylin/eosin (HE). The tissue and crystals were examined using a light microscope and a polarizing microscope.

Transmission electron microscope (TEM)

Electron microscopy was performed on kidney tissue obtained from rats treated with melamine and cyanuric acid for 4 weeks. Briefly, kidney tissue samples were fixed at room temperature in 2.5 % glutaraldehyde and cut into thin sections. After thorough washing in PBS, the samples were exposed to 1 % osmium tetroxide, dehydrated through a series of graded ethanol concentrations to 100 % ethanol, passed through two changes of propylene oxide, and embedded in Epon 812 for 24 h. Sections were then polymerized and stained. The structure of renal tubular epithelial cells was analyzed with H-7650 transmission electron microscope (Hitachi, Japan).

RNA preparation

Total RNA was extracted from tissues using TransZol kit (TransGen Biotech, Beijing). Whole tissue samples were homogenized in TransZol using a homogenizer (SONICS; Vibra cell, USA). Total RNA was treated with DNase I for 30 min at 37 °C followed by phenol chloroform extraction to avoid genomic DNA contamination. RNA concentrations were quantified spectrophotometrically at 260 nm. RNA integrity was verified by ethidium bromide staining of 28 S and 18 S rRNA after agarose gel electrophoresis. Final samples were stored at 80 °C until required for semiquantitative RT-PCR.

Semiquantitative RT-PCR

To determine monocyte chemoattractant protein-1 (MCP-1) mRNA expression in kidney, semiquantitative RT-PCR assay was performed by previously described methods [11]. Briefly, first strand cDNA was synthesized from 2 μg of total RNA using EasyScript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing). The subsequent PCR reaction was performed. The endogenous control, glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), was used to normalize the variations in the cDNA quantities from different samples. The primers were as follows: MCP-1 sense 5′-GTTGTTCACAGTTGCTGC CT-3′, MCP-1 antisense 5′-CTCTGTCATACTGGTCACT TCTAC-3′, GAPDH sense 5′-GCAAGTTCAACG GCAC AGTCAAGGCTG-3′, GAPDH antisense 5′-GCCCAGGA TGCCCTTTAGT GGGCCCTC-3′. The final reaction volume was 50 μl. Each sample was analyzed in triplicate. Levels of MCP-1 mRNA expression were assessed by comparing the density of bands relative to GAPDH on 2 % agarose gels.

TUNEL assay

Apoptotic cells were examined by terminal dideoxynucleotidetransferase-mediated X-dUTP nick end labeling (TUNEL) method using an in situ apoptosis detection kit (Takara Biomedicals) according to the manufacturer's instructions. Briefly, kidney tissue sections were deparaffinized and rehydrated, and treated with 20 μg/ml proteinase K at room temperature for 15 min. 3 % hydrogen peroxide inactivated the endogenous peroxidase. Sections were reacted with terminal deoxynucleotidyl transferase (Takara Biomedicals) and fluorescein isothiocyanate-deoxyuridine triphosphate (Takara Biomedicals) for 90 min at 37 °C in a humidified chamber. The tissues were viewed under a confocal laser scanning microscope (Leica, Heidelberg, Germany).The presence of a clear nuclear staining showed apoptotic cells.

Statistical analysis

Statistical analysis was performed with an independent *t* test using SPSS statistical software (version 10.0; SPSS Inc., Chicago, IL, USA). A P value of ≤ 0.05 was considered to be significant.

Results

Histological observations

The kidneys of the treatment groups were observed widely to be brownish-yellow precipitate on the surface at necropsy. Histological examination in kidney sections revealed that crystals were distributed widely in the cortex and medulla region, and rarely in the renal papillae (Fig. 1). MRCs may form in glomerulus and renal tubule segments including PCT, DCT, the limb loops of Henle and MCD in the cortex and medulla area (Fig. 2). Most crystals formed large aggregation in tubules, which damaged tubular epithelial structure and lead to tubular lumen dilatation and cell necrosis. The shapes of crystals were diversified. Irregular crystals were observed to aggregate in DCT and MCD (Fig. 2a, c). Emanative fan-shaped crystals could be found in the thin limb loops of Henle (TLH) distributed in the medullary area, and were large enough to block tubular flow (Fig. 2a, b). A small number of crystals were also detected in renal interstitium (Fig. 2a, c).

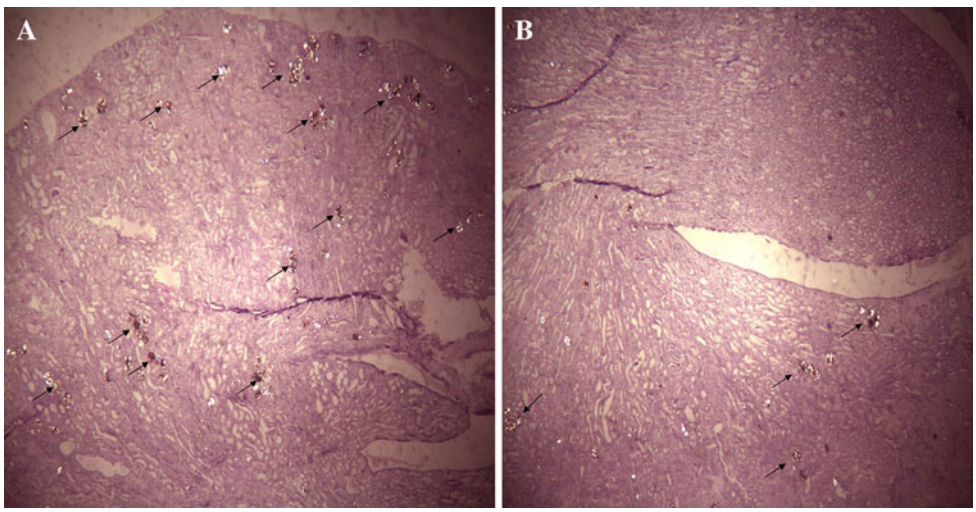

Fig. 1 Distribution of melamine-associated crystals in rat kidney. Crystals (*black arrow*) were observed mostly in the cortex and medulla area (**a**), and rarely in renal papillae (**b**). Magnification: ×40

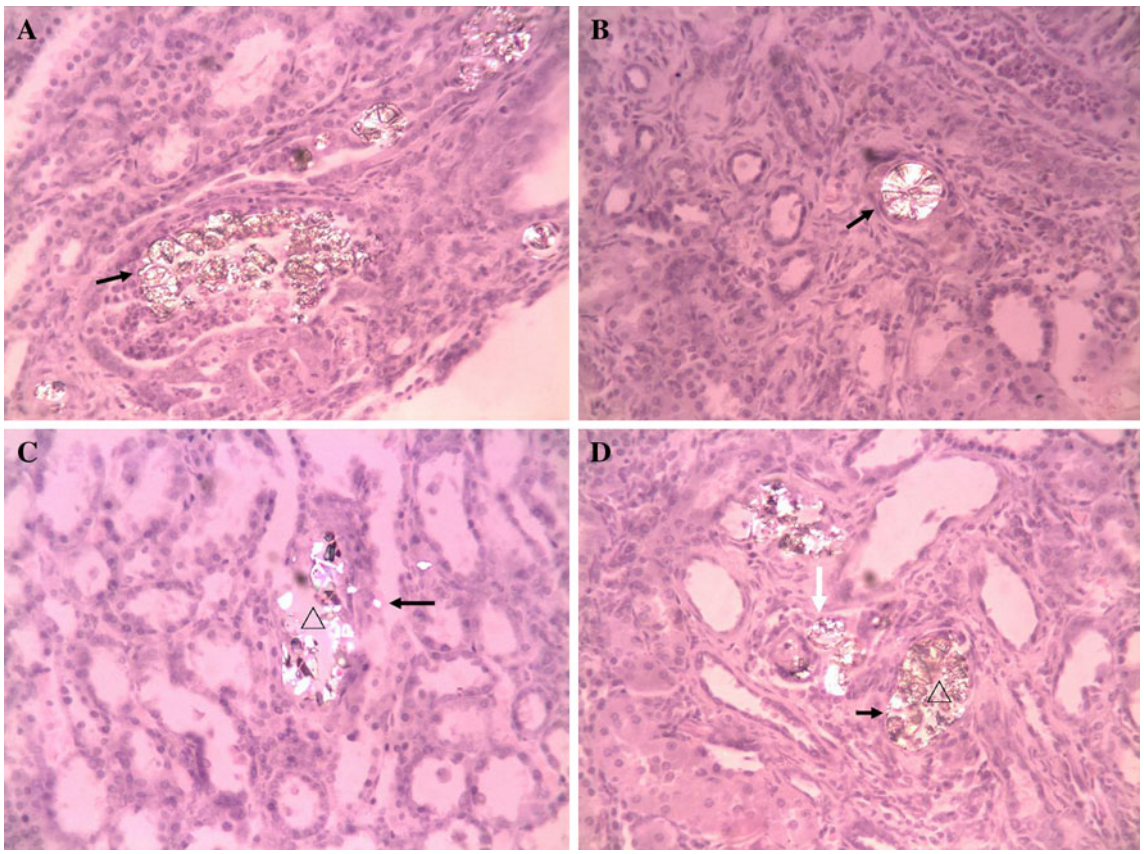

Fig. 2 Histological observations of melamine-associated crystals. Crystals formed in glomerulus (*white arrow*, **d**) and renal tubule segments in the cortex and medulla. The shape of crystals was diversified and including irregular crystals (*black arrow*, **a**) and emanative fan-shaped crystals (*black arrow*, **b**). Crystals lead to tubular lumen dilatation and tubular epithelial cell necrosis (*triangle*, **c**, **d**). A small number of crystals were also detected in renal interstitium (*black arrow*, **c**). Magnification: ×200

TEM observations

Renal tubular epithelia cells were examined by transmission electron microscope. It was observed that nuclear membrane shrunk, chromatin condensed under the nuclear membrane (Fig. 3b), lysosomal-related structures increased (Fig. 3c). More observedly, renal tubules epithelial cells disintegrated, numerous cytoplasmic vacuoles formed and the basement membrane broke, and the integrality of renal tubule was destroyed (Fig. 3d). The surrounding cells were oppressed and out of shape. Apoptotic bodies containing organelles or nuclear material were found in tubular lumen.

MCP-1 expression

MCP-1 is a potent agonist for mononuclear leukocytes and recruits monocytes to sites of inflammation in various pathological conditions, including atherosclerosis and renal epithelial cells on exposure to calcium oxalate, phosphate and uric acid crystals [10–13]. Figure 4 shows MCP-1 mRNA expression in kidney tissue in the present study. The MCP-1 mRNA expression was significantly increased in treatment rats (0.33 ± 0.22) compared with the control rats (0.08 ± 0.04, $P = 0.025$). This result revealed that inflammatory responses were involved in the progress of melamine-related kidney stone formation.

TUNEL assay

Apoptotic renal tubular cells can promote crystallization by providing substrates for crystals heterogeneous nucleation. Figure 5 shows an apoptotic condition in the kidney. In the treatment rats, apoptotic cells were presented widely in cortex and medulla, and their number was significantly more than that in the control group. In the medulla of treatment rats, clusters of apoptotic cells were found in tubule lumina and renal interstitium. However, there was no significantly observed difference of apoptosis in renal papillae between the treatment rats group and control rats.

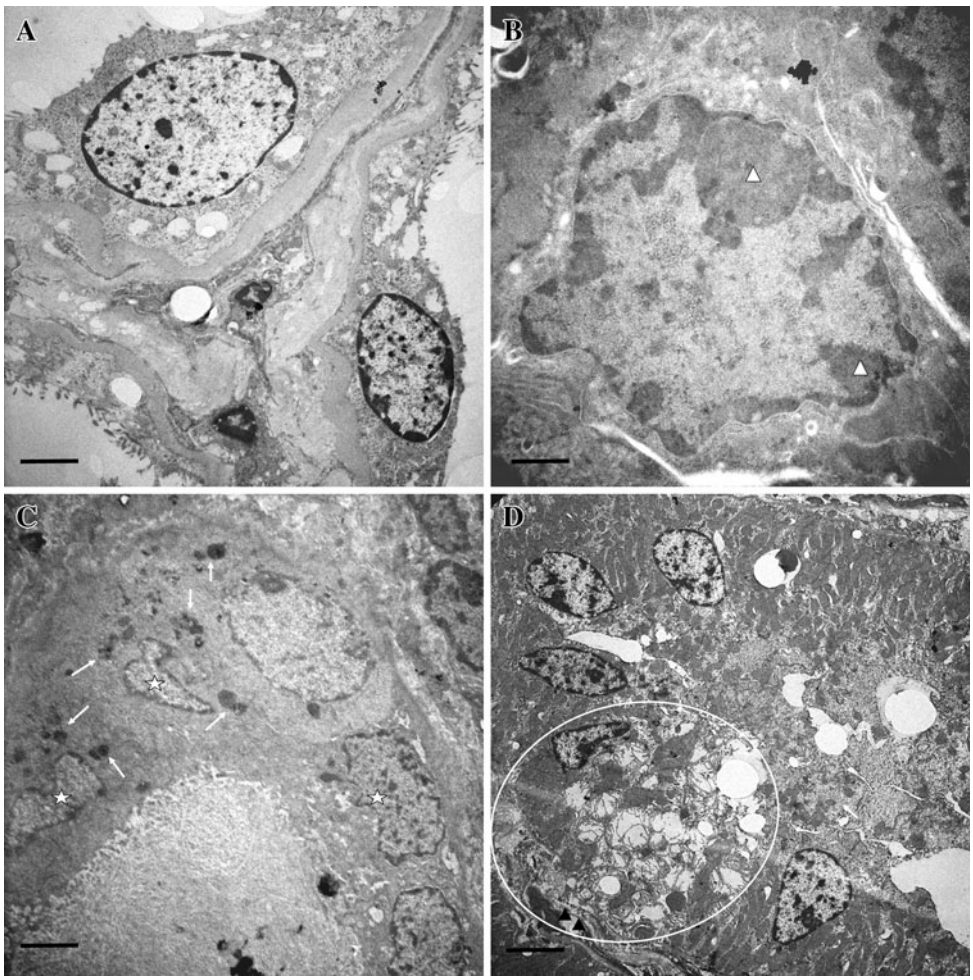

Fig. 3 TEM observations of renal tubular epithelial cell exposure to melamine and cyanuric acid. **a** Showed a normal rat kidney cell structure. *Scale bar* 2 μm. **b** Showed chromatin condensed under the nuclear membrane of the renal tubular cell (*white triangle*). *Scale bar* 1 μm. **c** Showed irregular and shrunk cell nucleus (*white star*), and increased lysosomal-related structures (*white arrow*). *Scale bar* 4 μm. **d** Shows the structure of normal renal tubule epithelial cell disappear and cells disintegrated, numerous cytoplasmic vacuoles formed and the basement membrane broke, the integrality of renal tubule was deficient (*white circle*). The surrounding cells were oppressed and out of shape. Apoptotic body containing organelles or nuclear material could be found in tubular lumen (*white arrow*). *Scale bar* 4 μm. Magnification: ×6,000–12,000

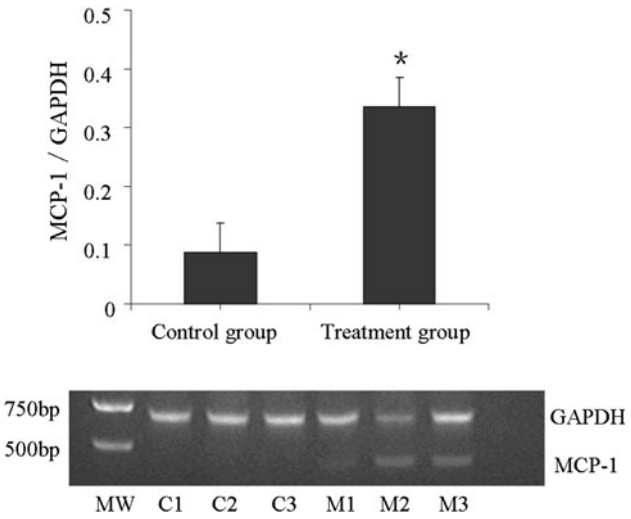

Fig. 4 Renal MCP-1 expression in rat administrated melamine and cyanuric acid. The MCP-1 mRNA expression was significantly increased in treatment rats compared with the control rats. $*P < 0.05$ versus control

Discussion

The pathogenesis of kidney stone was too complicated to understand by a single theory. As a new type of kidney stone, the formation mechanism of melamine-related kidney stone was more unclear. Previous studies showed that melamine or cyanurate acid alone failed to cause kidney stone, but combination of both might form melamine-related stone in kidney in cats or pigs [5, 14]. However, as by-products of melamine, levels of cyanuric acid in foods have not been systematically tested. In the present study, we performed an in vivo study to clarify the effect of melamine and cyanuric acid on renal tubules. The mixture of melamine and cyanuric acid with a 1:1 ratio was added into food for rats, and MRCs were induced successfully in the rats' kidney. Major findings were as follows: (1) MRCs may form in glomerulus and in a wide range of renal tubule segment, and may be large enough to block tubular flow; (2) renal tubular epithelial cells apoptosis, necrosis and inflammation were involved in melamine-related stone formation.

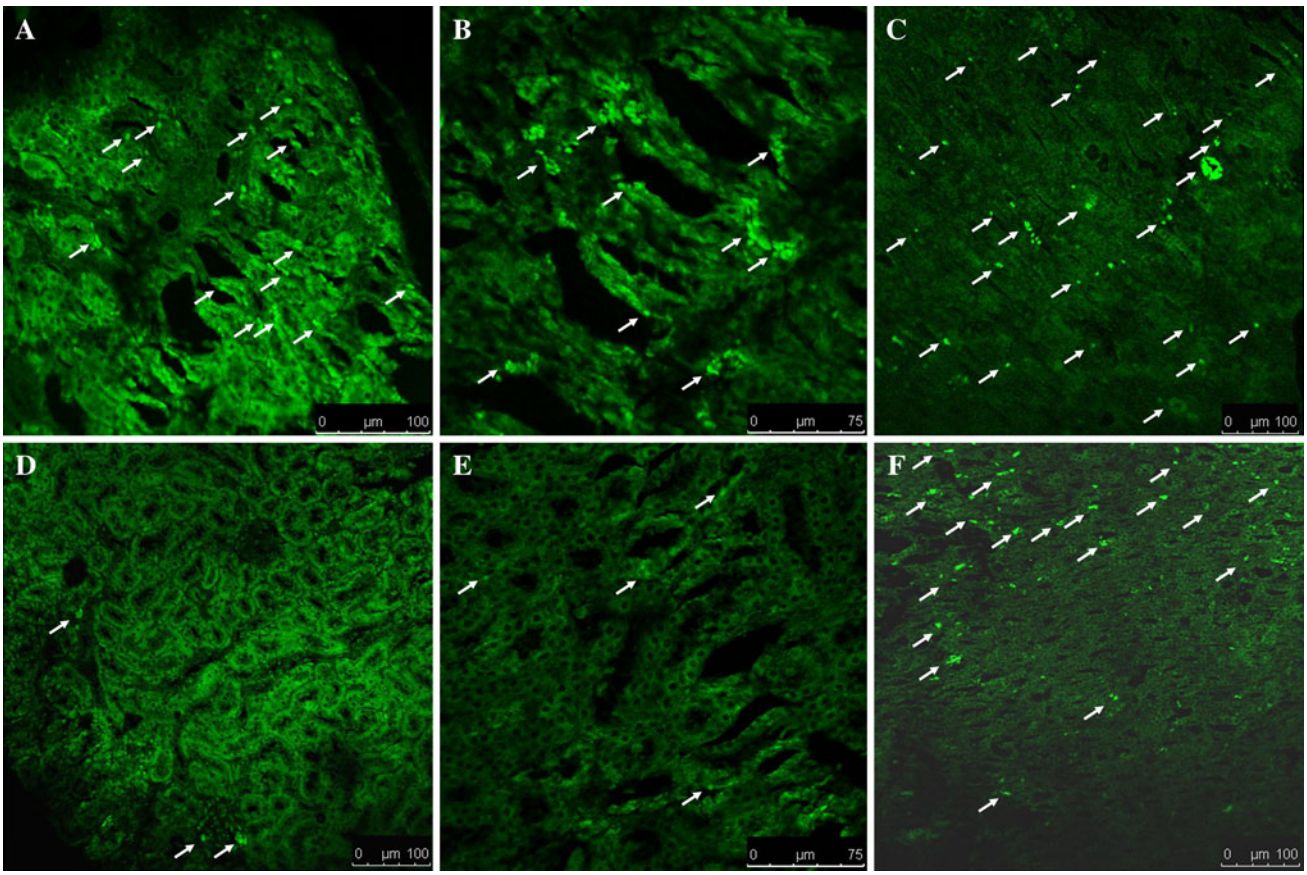

Fig. 5 Apoptosis observation in rat kidney. A clear nuclear staining (*white arrow*) showed apoptotic cells. Apoptotic cells were showed in the cortex (**a**), medullary (**b**) and papillae (**c**) in the treatment rat kidney, and in the cortex (**d**), medullary (**e**) and papillae (**f**) in the control rat kidney

Several characteristics of MRCs were noticed. Firstly, it was markedly different from common oxalate calcium crystals formation induced by hypercalciuria or hyperoxaluria. MRCs were found to be located in a wide renal tubular segment in the cortex and medulla and rarely in the renal papilla where oxalate calcium crystals easily deposited. This finding was consistent with the recent report in low and high-dose melamine and cyanuric acid administered rats by Takahiro et al. [15]. Secondly, MRCs were mostly found to form large crystal aggregations in tubules. This implied that it was easier for MRCs to press renal tubular epithelial cell and lead to epithelial cell injury. On the other hand, it was also easier to obstruct tubular flow and break through injured renal tubule epithelial cells into renal interstitium. This finding was consistent with a previous study on dogs and cats affected by pet-food contaminated with melamine [16]. The profiles of tubules were dilated both in the presence and absence of associated crystals, fewer epithelial nuclei were present, epithelial cells were attenuated. Thirdly, MRCs were capable of forming in glomerulus, where there was no evidence of oxalate calcium crystals formation. To our knowledge, it is the first finding that MRCs form in glomerulus in rat. We considered that proteins in blood may inhibit MRCs formation. Once these proteins are filtrated from glomerulus, their inhibiting action is weakened and MRCs formed. This hypothesis was supported by the fact that there was no crystal formation in the blood vessel. The above characteristics of MRCs suggested that melamine-related stone formation might have a different mechanism from that of oxalate calcium crystals, and be severer in renal dysfunction than common calcium oxalate stone.

Crystal formation is as a result of a cascade of events, including crystal nucleation, growth, aggregation, and crystal retention within the renal tubules [17]. Renal tubular epithelial cells injury and apoptosis can promote crystallization by providing substrates for crystals heterogeneous nucleation. Cell degradation following renal epithelial injury produces numerous membrane vesicles, which are good nucleators of crystals, and enhances crystal nucleation at low supersaturation and promotes crystal–cell interaction [18]. Our results showed that MRCs cause renal tubular injury and apoptosis, even result in loss of the integrality of renal tubule, and crystals may break through defective renal tubule into the interstitium. These findings were similar to those observed in the dogs and cats in the

United States in 2007 [16]. In the affected dogs and cats, tubular necrosis and renal interstitial fibrosis and lymphoplasmacytic inflammation were observed. Our findings suggested that renal tubular cells injury and apoptosis may play a significant role in forming melamine-related kidney stone.

We observed a significantly increased MCP-1 expression in the kidney of MRCs forming rats. Directed migration of leukocytes in response to inflammatory stimuli is crucial for the cellular and adaptive immune response. Kidney cells produce MCP-1 in response to various proinflammatory stimuli [19], and its expression has been predictably identified in kidney diseases which involve significant inflammation [20]. Previous study found that renal macrophage migration and crystal phagocytosis were associated with inflammatory-related gene expression during calcium oxalate kidney stone formation by Okada et al. [21]. Our finding indicated that melamine and cyanuric acid would stimulate inflammatory responses in the rat kidneys. In the previous study on dogs and cats affected by pet-food contaminated with melamine, lymphoplasmacytic inflammation, consisted of moderate numbers of lymphocytes, plasma cells, macrophages, and only rare neutrophils, was found to surround crystal-containing tubules [16]. Inflammatory responses may also be key regulators of development of melamine-related kidney stone.

In summary, our study showed that melamine and cyanuric acid will lead to renal tubular epithelial cell injury, apoptosis and inflammation, which may play a promoting role in forming melamine-related kidney stone. These definite conclusions will be validated by a larger number of studies in the future. Our findings are important for understanding pathogenesis of melamine-associated kidney stone formation and estimating its clinical prognosis.

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